Surfactants produced by epiphytic bacteria and their role in diesel degradation

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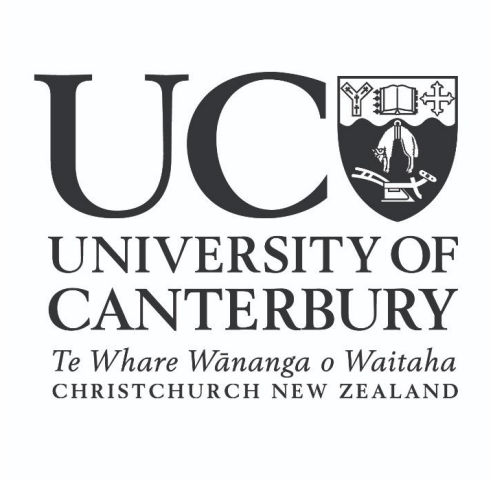
A thesis submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

in Microbiology

at the University of Canterbury

by Simisola O. Oso



2021

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Abstract

Hydrocarbon contaminations cause serious harm in the environment. Bacterial bioremediation is an environmentally friendly method to reduce such contaminants. To increase the bioavailability of hydrocarbons, bacteria produce surfactants. Many leaf colonising bacteria are surfactant producers but have not been tested for their potential to remediate hydrocarbon contaminations. The aim of this thesis was to investigate if surfactants produced by leaf colonising bacteria enhance the degradation of hydrocarbons in terrestrial environments.

A high proportion of the bacterial strains investigated in this thesis was found to produce surfactants and degrade diesel in liquid culture. Furthermore, four leaf isolates of Pseudomonas and their surfactant mutants were used to study the effect of surfactants on diesel degradation and colonisation on Arabidopsis thaliana leaves. No differences in the ability to colonise leaves were observed when comparing the wild type with its mutant derivatives. However, when tested in liquid cultures, all mutants were found to grow slower on minimal medium supplemented with diesel. This effect was complemented by the addition of wild type or synthetic surfactants to the growth medium. This evidences the role of surfactants in enhancing diesel bioavailability in liquid environments. By contrast, the same bacterial strains did not exhibit significant growth differences in diesel contaminated soil microcosms. Wild types and mutants were equally able to grow and degrade diesel as determined by gas chromatography coupled with flame ionisation detection.

In conclusion, many epiphytic bacteria are hydrocarbon degraders and should be investigated further as potential candidates for hydrocarbon degradation in soil. Furthermore, in Pseudomonads, surfactant production does impact diesel degradation in liquid but not in soil.

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Please detail the nature and extent (%) of contribution by the candidate
100% of the laboratory work was performed by and the first manuscript draft was written by the candidate. Walters provided assistance during the establishment of the photographic methods for data acquisition. Schlechter produced the phylogenetic tree. Remus-Emsermann gave feedback and commented on the manuscript.

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Please detail the nature and extent (%) of contribution by the candidate:

The candidate has performed all growth experiments, strain characterisation, data acquisition and analysis published in the paper and co-drafted the manuscript in total ~80% of the work. Fuchs, Übermuth, Zander, Daunaraviciute produced surfactant knockout mutants. Fuchs purified surfactants for MS-MS analysis. Remus determined transposon insertion sites. Stötzel and Wüst performed the MS-MS analysis. Schreiber supervised and led the project. Remus-Emsermann supervised and led the project and drafted the manuscript with the candidate.

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1 Introduction

1.1 Background

Petroleum hydrocarbons are complex mixtures of alkanes, asphaltenes, aromatics and Fresins (Abbasian et al. 2015). Pollution from petroleum hydrocarbons poses great hazards in the aquatic and terrestrial environments (Koshlaf et al. 2017). Due to the persistent nature of these hydrocarbons, they can remain in the environment for a long-time affecting soil quality by altering physical and biochemical properties, and the indigenous microorganisms inhabiting the soil (Holliger et al. 1997).

There are various methods of treating hydrocarbons, including physical, chemical and thermal treatments (Das and Chandran 2011; Zeng 2000). These treatments are expensive and may require secondary treatments. It is, therefore, important to find environmentally friendly and cost-effective methods of treating pollutants arising from the use of petroleum hydrocarbons (Riser-Roberts 1992). Microorganisms have been used over the years as a remediation method of choice in the degradation of hydrocarbons (Varjani, 2017). During remediation, petroleum hydrocarbons are converted to CO_2, H_2O and biomass which are less harmful in the environment (Das and Chandran 2011).

For degradation of hydrocarbons to occur, the first requirement involves bacterial membrane oxygenases but these require direct contact between substrate. Factors such as the bioavailability, the concentration of the hydrocarbon and environmental factors also contribute to the success of degradation of hydrocarbons by naturally occurring microorganisms. The degradation of petroleum hydrocarbons is carried out by an enzyme system, attachment of microbial cells to the hydrocarbon or by the production of surfactants (Das and Chandran 2011; Xu et al. 2018). The utilisation of surfactant producing bacteria in the degradation of hydrocarbons is a method of choice in the degradation of hydrocarbons (Das and Chadran 2010). However, the utilisation of surfactant producing bacteria in the degradation of hydrocarbons depends on the type of hydrocarbon, physiological

characteristics of the bacteria and the type of surfactant produced by the bacteria (Hua and Wang 2014).

The phyllosphere, which is discussed in detail below, is habitat to a wide range of microorganisms, with bacteria being the most abundant (Remus-Emsermann and Schlechter 2018). Microorganisms present on plant leaf surfaces are able to produce surfactants which assist in their effective survival and exploration of the phyllosphere (Bunster, Fokkema, and Schippers 1989; Lindow and Brandl 2003). The surfactants produced by these epiphytic bacteria have found uses in the agricultural, pharmaceutical and cosmetic industries (Deleu and Paquot 2004; Gudiña et al. 2013). Surfactants produced by epiphytic bacteria enhance the removal of organic compounds such as petroleum hydrocarbons by decreasing surface and interfacial tensions as further discussed in subsequent chapters of the thesis (Bai et al. 1997).

The surfactant-producing epiphytic bacteria possess hydrocarbon degrading genes and are capable of degrading hydrocarbons (Xu et al 2018). However, it is not clear if these epiphytic bacteria gain fitness by degrading the hydrocarbons present on plant leaf surfaces (Vorholt 2012).

1.2 The phyllosphere

The above ground surface of plants, composed of leaves, stem, flowers and fruits is known as the phyllosphere (Ruinen 1956; Müller and Riederer 2005). The phyllosphere is dominated by leaves. Leaves are covered by a hydrophobic cuticle (Figure 1-1) (Remus-Emsermann and Schlechter 2018). The cuticle protects against water loss, dissolution of organic chemicals by transpiration (Riederer and Schreiber 2001), and protection against pathogen and insect attack (Eigenbrode and Jetter 2002; Serrano et al. 2014). The cuticle also reduces the effect of UV radiation (Krajšek et al. 2011), and offers protection against chemical and mechanical injury (Heredia 2003; Yeats and Rose 2013; Kim, Choi, and Suh 2017). Cuticles vary in composition, structure and thickness depending on the plant species.

The cuticle is composed of a wide range of compounds with different physicochemical properties (Ridge 1997; Barthlott et al. 1998). These compounds include biopolyester cutin, waxes, phenolics, mineral elements and polysaccharides.

The biopolyester cutin is mainly composed of C_{16} and C_{18} hydroxy and hydroxy epoxy fatty acids as well as saturated and unsaturated fatty acids, small amount of glycerol, di-carboxylic acids and phenyl-propanoids (Heredia 2003; Wattendorff and Holloway 1980; Stark and Tian, 2006; Pollard et al. 2008). The major components of cutin are 16-hydroxy and dihydroxyhexadecanoic acid which possess a single hydroxyl moiety at the terminal carbon and another in the mid chain position. The C_{18} family on the other hand possesses an unsubstituted 18-hydroxy octadec-9-enoic acids, 18-hydroxy-9-epoxy and 9,10,18-trihydroxyoctadecanoic acids. The monomers are crosslinked by esters. Based on its chemical composition, the cuticle can be divided into two layers: the cuticular layer proper i.e. the rich in polysaccharides and an overlying layer with less polysaccharide but an abundance of waxes (Yeats and Rose 2013).

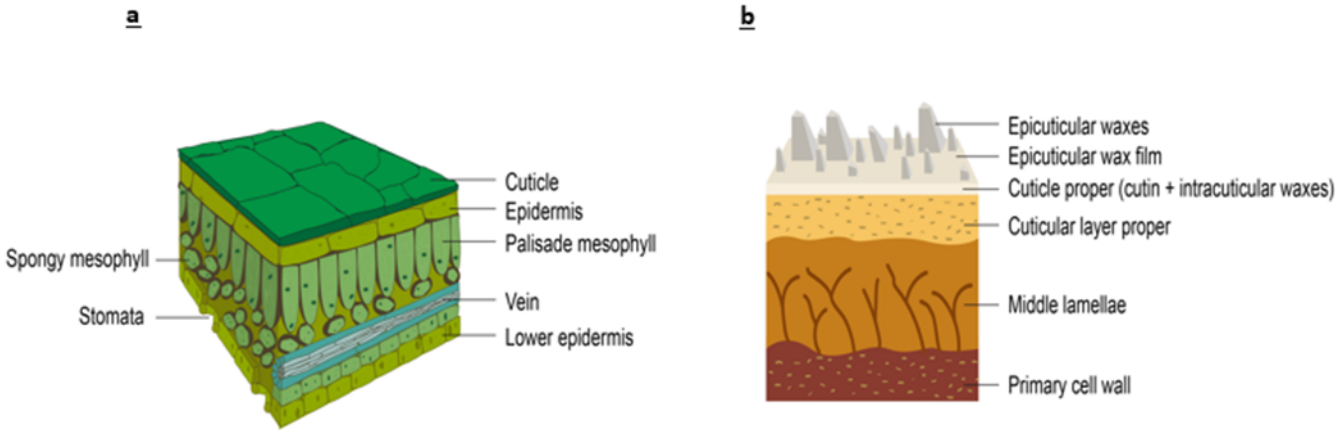


Figure 1-1. Schematic representation of the plant leaf surface showing the waxy cuticle. (a) The cuticle covers the epidermis which reduces the rate of water loss. The palisade mesophyll includes tissues which are responsible for photosynthesis in most plants. The vascular bundle provides support for the leaf, enhancing water and solute transport. (b) The structural features of the cuticle. Adapted after Holloway, 1994.

The cuticle gains most of its physicochemical properties such as its hydrophobicity and optical appearance from cuticular waxes (Schmidt and Schönherr 1982; Riederer and

Schönherr 1988; Buschhaus and Jetter 2011; Zeisler and Schreiber 2016). The cuticular waxes embedded in the cuticle are known as the intracuticular waxes while the waxes superimposed onto the cuticle are the epicuticular waxes. They are differentiated by their chemical composition, ultrastructure and development. Plant cuticular waxes are composed of major compounds such as n-alkanes, primary alcohols, fatty acids, aldehydes, secondaryalcohols, ketones and n-alkyl esters formed from long-chain primary alcohols and fatty acids (Jetter, Kunst, and Lacey Samuels, 2006).

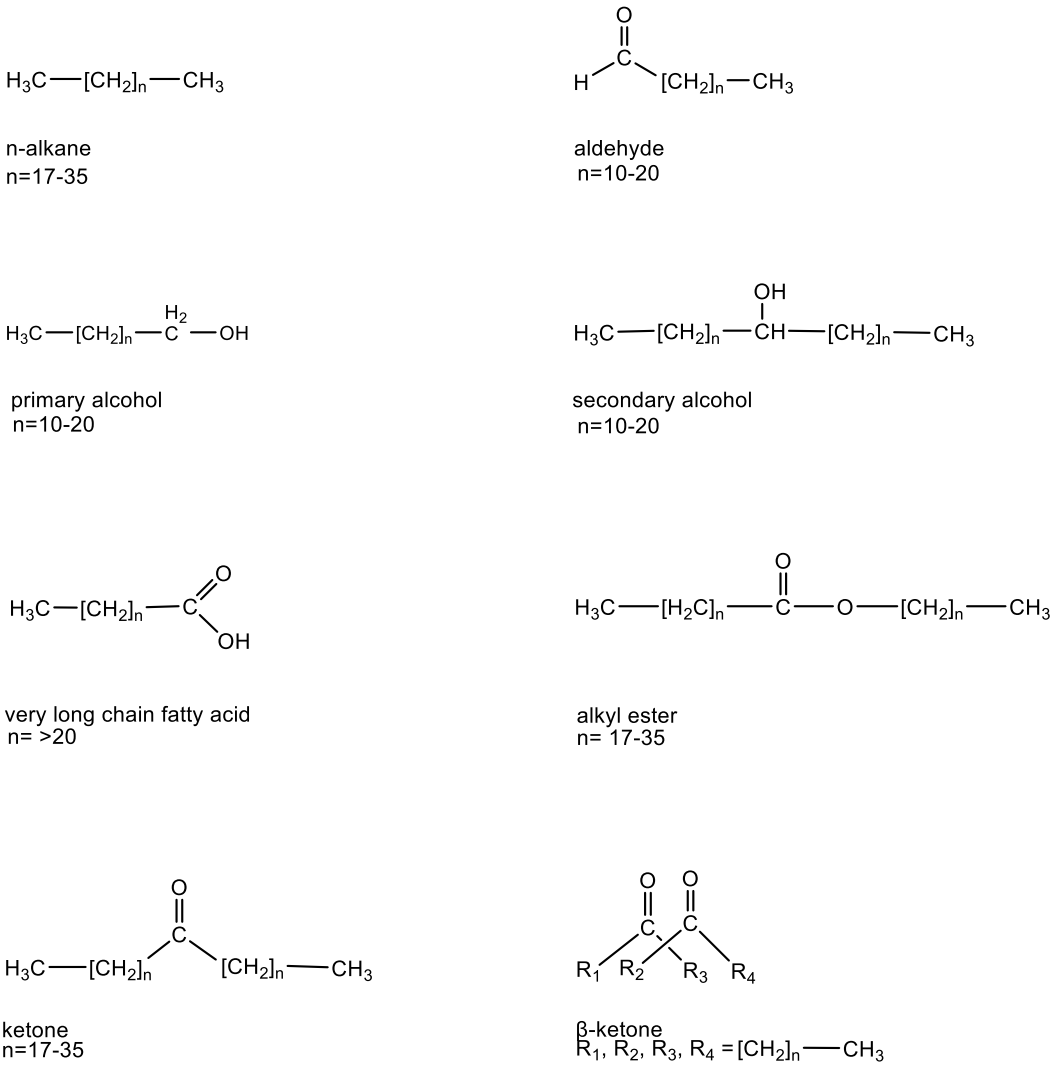


Figure 1-2. Components of the cuticular wax: n-alkane, aldehyde, primary alcohol, secondary alcohol, very long chain fatty acid, alkyl ester, -diketone and ketone. The epicuticular waxes are a mixture of aliphatic hydrocarbons with a variety of substituted groups. The n-alkanes range in chain length from C_{17}-C_{35} while the R_1 and R_2 contain up to C_{10}-C_{20}. The hydrocarbon chains may contain substituted group in mid chain i.e. alkyl esters, ketones ß-ketones and di-ketones or in terminal positions i.e. very long chain fatty acids, primary alcohols and aldehydes. Adapted from Jacquemond and Ustin, 2019.

1.3 Microorganisms in the phyllosphere

A wide range of microorganisms i.e. fungi, algae, yeasts and bacteria colonise the plant leaf surface. Bacteria, however, are the most abundant ranging from 10^4-10^5 bacteria/mm^2 (Remus-Emsermann and Schlechter 2018). Examples of different phyla inhabiting the phyllosphere are Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Remus-Emsermann and Vorholt 2014). The population of these bacteria is highly dependent on environmental factors such as nutrient availability, humidity, temperature, pollution, and UV-radiation (Vorholt, 2012). Factors including host's genotype and geographical location also affect bacterial composition and variability on the plant leaf surface (Kinkel, Wilson, and Lindow 2000; Vorholt 2012).

Environmental conditions impact on the colonisation of leaf surfaces of low relative humidity have shown to decrease or change the bacterial population on leaves (O'Brien and Lindow, 1989; Hirano and Upper, 2000). Also, weather conditions can determine the most abundant most abundant colonisers of leaves. For example, *Pseudomonas syringae* was abundant during the wet and warm weather periods while pink-pigmented facultative methylotrophs (PPFMs) were most abundant during the dry and hot weather (Hirano and Upper, 2000). Rain is also known to cause an increase in the number of epiphytic bacteria on the phyllosphere (Hirano and Upper, 2000). Others have shown that exposure of epiphytic bacteria to changes in UV affect the population of bacteria inhabiting the phyllosphere (Newsham et al. 1997; Sundin and Jacobs, 1999; Hughes et al. 2003). For example, phyllosphere exposed to UV showed an increase bacterial diversity (Kadivar and Stapleton, 2003). Leaf age is also a major factor in determining the population of microorganisms in the phyllosphere (Copeland et al., 2015; Redford and Fierer, 2009; Maignien et al., 2014). Microorganisms such as bacteria, filamentous fungi and yeasts can colonise the same leaf surface, a succession in microbial composition as the leaf ages (Leveau, 2006).

The availability of carbon containing compounds which are sugars, nitrogen sources and other essential inorganic molecules play major roles in terms of colonisation of

microorganisms in the phyllosphere (Wilson and Lindow 1994; Mercier and Lindow, 2000). Epiphytic bacteria can alter the phyllosphere to access nutrients. For example, some *Pseudomonas* spp. can increase the wettability of plant leaf surfaces by producing surfactants which may help their effective survival in the phyllosphere. (Bunster, Fokkema, and Schippers 1989; Lindow and Brandl 2003). When wettability is increased, the contact between water and the hydrophobic plant leaf surface is enhanced. The production of surfactant by epiphytic bacteria also increases the spread of water which results in increased elevated water content enabling the movement of water and soluble nutrients to the leaf surface thereby promoting bacterial growth (Beattie 2011; Bunster, Fokkema, and Schippers 1989; Knoll and Schreiber 2000; Schreiber et al. 2005; Hess, Dan Hess, and Foy 2000). Surfactants might therefore allow microorganisms to exploit the phyllosphere more effectively (Bunster, Fokkema, and Schippers 1989; Lindow and Brandl 2003).

1.4 Petroleum as a major source of contaminant

Aside from surfactants produced by bacteria performing the roles mentioned above on leaf surfaces, surfactants are also important for the degradation of hydrocarbons. The use of surfactant-producing microbes enhances the effectiveness of hydrocarbon degradation as the surfactant plays an important role in enhancing the rate of dissolution or desorption leading to the solubilization or emulsification of petroleum hydrocarbon pollutants (Patowary et al. 2017; Varjani and Upasani 2017).

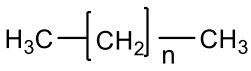
Petroleum contains a complex mixture of hydrocarbon, and nitrogen, sulphur and oxygen. Petroleum components are formed by thermal decay of organic compounds over geological time scales (Xu *et al*., 2006). Petroleum constituents include aromatics, asphaltenes, the saturates and resins (Abbasian et al. 2015). The saturates are hydrocarbons with no double bonds and are grouped according to their chemical structures. They represent the largest relative proportion of petroleum (Chandra et al. 2013; Abbasian et al. 2015) and are primarily represented by alkanes. Their structures can either be branched, open-chain or linear.

Representatives of these structures are the n-alkanes, iso-alkanes and cycloalkanes e.g. naphthene. There are four molecular weight groups of n-alkanes. These are gaseous alkanes (>C_8), low molecular weight alkanes (C_8-C_{16}), intermediate molecular weight alkanes (C_{17}-C_{28}), and high molecular weight alkanes (>C_{28}) (Abbasian et al. 2015; Varjani 2017).

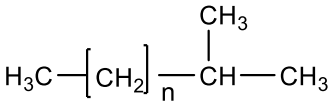
The chemical structures of petroleum hydrocarbons may affect biodegradation in two ways: (i) the molecules contain groups that cannot react with available or specific enzymes involved in hydrocarbon degradation; and (ii) the structure of the hydrocarbon to be degraded may be in a physical state where degradation does not occur easily (Das and Chandran, 2010). It has been shown that the more complex a hydrocarbon is, the more difficult it is to be degraded (Kanaly and Harayama, 2000).

In this thesis, diesel was used as the model hydrocarbon due to its availability at the time the study was conducted. Diesel is commonly used in engines with high rotation speed. The number of carbon chain in diesel can range from C_{11}-C_{25} with distillation range of 180-380° C (Ahmed and Fakhuruddin, 2018). Diesel can be categorised into saturated, unsaturated and aromatics fractions which will be explained below (Figure 1-3) (Logeshwaran et al., 2018; Speight et al., 2015). The saturated hydrocarbons have single bonded carbon atoms while the unsaturated hydrocarbons have one or more double or triple bonded carbon atoms. The aromatic fraction contains more than one aromatic ring that maybe linked to substituted rings (Logeshwaran et al., 2018). These classes are further divided into alkanes, alkenes and aromatics such as naphthene (Logeshwaran et al., 2018) (Figure 1-3). There are 2000 - 4000 hydrocarbon compounds in diesel with 70% alkanes and 30% aromatics (Imron et al.,2020).

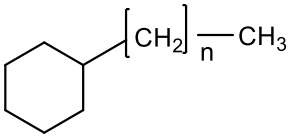
A



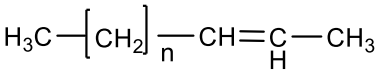
n-alkanes
n= 6-26



i-alkanes
n=1-15

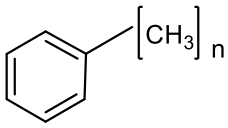


cycloalkanes
n= 5-9



alkenes
n= 5-20

B



aromatics
n=0 (benzene)

Figure 1-3. Structural representatives of hydrocarbon compounds in diesel. The molecular composition of hydrocarbons present in diesel are the saturated, unsaturated and aromatics which are further divided into alkanes, alkenes and aromatics. (A) n-alkanes contain C_8-C_{28}, iso-alkanes contain C_6-C_{20}, cycloalkanes contain C_5-C_9, alkenes contain C_5-C_{20} while (B) aromatics contain C_1-C_{10}.

1.5 Degradation of hydrocarbons by bacteria

Biodegradation is a natural process that has harnessed microorganisms to clean up environmental contaminants. During the process of biodegradation, microorganisms utilize

hydrocarbon contaminants as sources of carbon and energy (Obayori *et al*., 2009) and degrade harmful environmental contaminants into less harmful compounds such as CO_2, CH_4 and H_2O and biomass (Mosa et al. 2016). Biodegradation is a method of bioremediation that is inexpensive and an effective method of treating hydrocarbon contamination as it eliminates the chance of future liability associated with the treatment and disposal of contaminants (Balba, Al-Awadhi, and Al-Daher 1998; Boopathy 2000; Vidali 2001; Bacosa and Inoue 2015).

Microorganisms such as fungi, algae and bacteria can degrade hydrocarbons. Bacteria are, however, the major hydrocarbon degraders (Bundy, Paton, and Campbell 2004). Bacterial genera that have been shown to aid the degradation of petroleum are *Achromobacter, Acinetobacter, Alkanindiges, Alteromonas, Arthrobacter, Brevibacterium, Burkholderia, Corynebacterium, Dietzia, Enterobacter, Flavobacterium, Gordonia, Kocuria, Marinobacter, Mycobacterium, Pandoraea, Pseudomonas, Staphylococcus, Streptobacillus, Streptococcus, and Rhodococcus* (Atlas and Bartha, 1992; Chaillan et al. 2004; Okoh and Trejo-Hernandez, 2006; Jin et al. 2012; Varjani 2017; Xu et al. 2017; Sarkar et al. 2017). However, biodegradation is only effective when environmental factors, such as temperature, pH, and nutrients, allow the growth and activities of these microorganisms that degrade the contaminants (Vidali 2001). Additionally, competition with other microorganisms and presence of substrate degraded in preference to petroleum hydrocarbons are also important factors to be considered during biodegradation (Wilson and Jones 1993).

Biodegradation of petroleum hydrocarbons is reported to be more effective when the environment contains a mixed culture of microorganisms when compared to an environment with pure cultures (Adebusoye et al. 2007; Mukred et al. 2008; K. Das and Mukherjee 2007; Mariano et al. 2008; Mukred et al. 2008; Janbandhu and Fulekar 2011). This is because a mixed culture of bacteria may possess a consortium of catabolic genes that together are required for accelerated degradation of hydrocarbons and which are usually not found in a

pure culture. Consequently, the interactions between the members of the mixed culture leads to complete degradation of the substrate (Janbandhu and Fulekar 2011).

Limited bioavailability of hydrocarbons and lack of cell contact between the bacteria and hydrocarbons are the major factors that affect the degradation of hydrocarbons. Bacteria are, however, able to degrade hydrocarbons by developing countermeasures such as improved adhesion ability of cells to alter their surface components and the production of bioemulsifiers which enhance the access to the hydrocarbon to be degraded (Krasowska and Sigler 2014; Xu et al. 2018). The adhesion of hydrocarbons to be degraded to the bacterial cell is accomplished by outer membrane proteins and lipids, capsules, fibrils and fimbriae and chemical compounds present on cell surfaces e.g. prodigiosin and gramicidin (Ron and Rosenberg 2014). Microbial adhesion to hydrocarbons is effective under conditions of low bioavailability, n-alkanes and polycyclic aromatic hydrocarbons. Degradation of hydrocarbon does not necessarily require the adherence of the bacterial cell to the hydrophobic phase (Rosenberg et al 1982). Hori et al. 2008; Watanabe et al. 2008 showed that the thin fimbriae of mutant strain of *Acinetobacter calcoaceticus* RAG-1 contribute in adherence to hydrocarbons and polystyrene.

Microorganisms are also able to enhance the degradation of hydrocarbons through surfactant-mediated uptake. Surfactants increase the solubility of hydrocarbons and, thereby increase the bioavailability to microorganisms (Abbasnezhad, Gray, and Foght 2011).

1.6 Metabolic pathways for the biodegradation of petroleum hydrocarbons

Microorganisms can utilize hydrocarbons to obtain carbon and energy (Cerniglia 1992; Obayori et al. 2009) in three different ways: i) aerobic, ii) anaerobic and iii) anoxygenic degradation (Varjani 2017). In the following section, the focus is on the aerobic degradation of hydrocarbons because the epiphytic bacteria used in this study are aerobic.

1.7 Aerobic degradation

When the complete mineralisation of petroleum hydrocarbons occurs, the result is the formation of CO_2 and water (Das and Chandran 2011). Microorganisms use a biodegradation pathway which transforms organic contaminants into intermediates of the central intermediary metabolism (Figure 1-4). For example, microorganisms can degrade n-alkanes with the aid of soluble or integral membrane non-haem iron mono-oxygenases. Alkane hydroxylases hydroxylate the substrate (Karigar and Rao 2011; Abbasian et al. 2015; Varjani 2017). During the aerobic degradation of alkanes, the initial process is the oxidation of the terminal methyl group which produces a primary alcohol. Products obtained during this process are then oxidised by alcohol and aldehyde dehydrogenases to yield a corresponding aldehyde, which is then finally converted to fatty acid through oxidation. The fatty acid is linked to CoA and is channelled into the β-oxidation pathway in the form of acetyl-CoA. Microorganisms can also degrade long chain alkanes via subterminal oxidation. Generated secondary alcohols are transformed to the corresponding ketone during subterminal oxidation. Products obtained are converted to an ester via an oxidation step via a Baeyer-Villiger monooxygenase which is then hydrolysed by an esterase to generate an alcohol and a fatty acid (Wentzel et al. 2007; Varjani 2017).

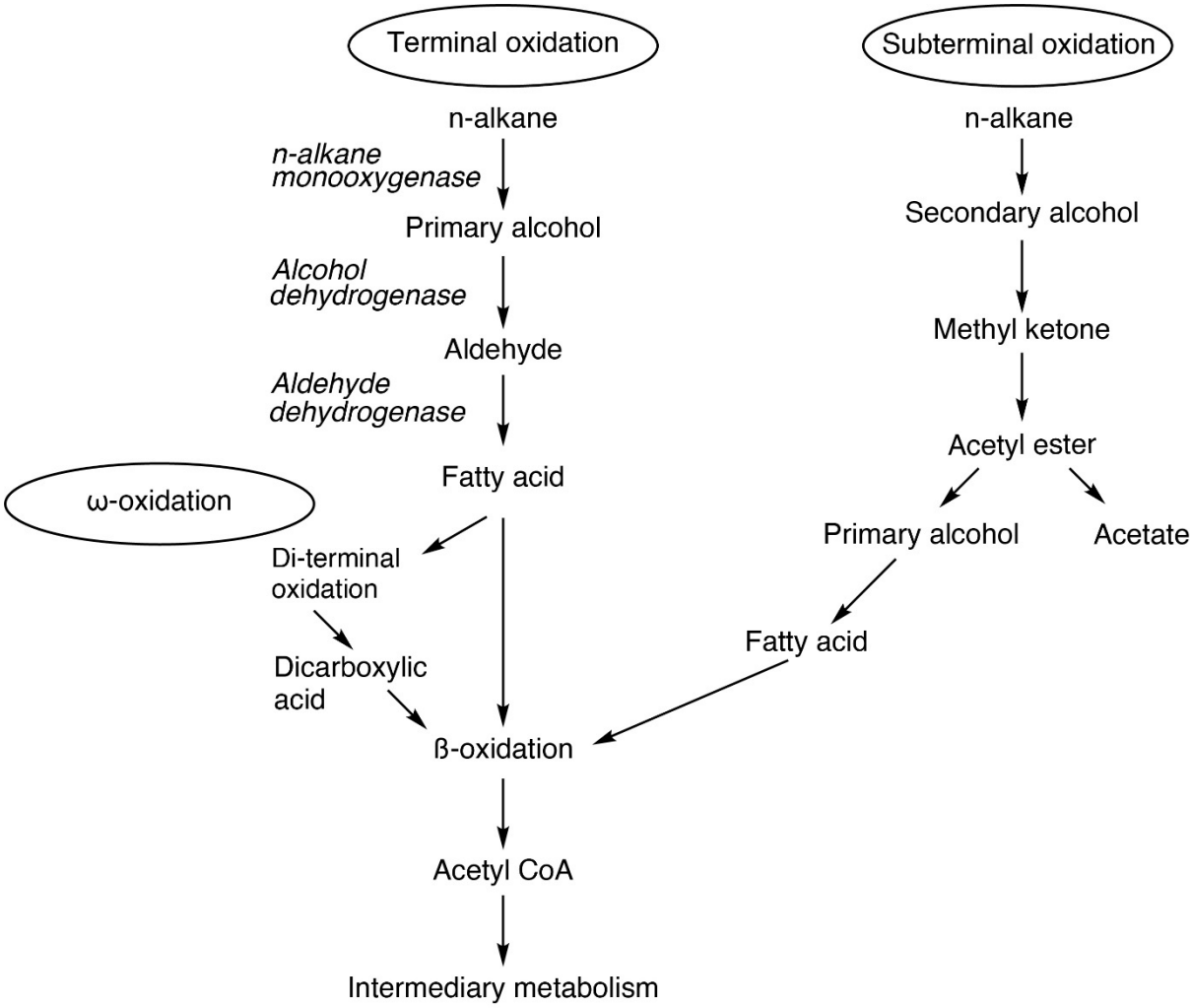


Figure 1-4. The three pathways for aerobic degradation of n-alkanes by bacteria. Terminal oxidation results in the formation of fatty acids which leads to ß-oxidation pathway. Alternatively, ω-oxidation by a fatty acid monooxygenase may occur resulting in dicarboxylic acids. The subterminal oxidation results in secondary alcohols which are oxidised to ketones. Adapted from Varjani, 2017.

1.8 Environmental factors that affect the biodegradation of petroleum hydrocarbons

Various environmental factors impact the effectiveness of biodegradation in soil. Factors such as temperature, oxygen content, nutrient content and soil pH should be considered in order to obtain optimal degradation of petroleum hydrocarbons (Johnsen, Wick, and Harms 2005). Temperature plays a significant role in the rate of degradation of hydrocarbons. Temperature not only affects the physico-chemical composition of the hydrocarbon, it also plays a significant role in bioavailability and also the metabolic activities of the microorganisms (Boopathy 2000; Das and Chandran 2011). Degradation of hydrocarbons has been observed over a wide range of temperatures, including psychrophilic, mesophilic

and thermophilic conditions (Prescott, Harley, and Klein 2005). However, the rate of petroleum hydrocarbon degradation has generally been found to decrease with decreasing temperature. This has been attributed to decreasing enzyme activity (Leahy and Colwell 1990), increased oil viscosity, reduced volatilisation of toxic short chain alkanes and increased water solubility leading to a delay in the start of the degradation process (Atlas and Atlas 2007). A temperature range of 20 - 30° C appears to be optimal for the degradation of petroleum hydrocarbons (Vidali 2001).

Oxygen content is also an important factor that impacts hydrocarbon degradation. Oxygen concentration determines if the environment operates aerobically or anaerobically. The metabolism of hydrocarbons which results in repeated oxidation of the hydrocarbon molecule is catalysed by enzymes. When this process is carried out, molecular oxygen acts as the electron acceptor. The amount of oxygen available in soil is dependent on moisture content, soil type and the rate of biodegradation (Wentzel et al. 2007).

The availability of nutrients, such as nitrogen, phosphorus and potassium, are determinants for both the growth of microorganisms and their ability to degrade petroleum hydrocarbons. Excess of these nutrients can inhibit the process of degradation of hydrocarbons while the absence or low levels of these nutrients can inhibit microbial activity and cell growth (Liebeg and Cutright 1999).

1.9 Enhancing bioremediation using biosurfactant-producing microorganisms.

The bioavailability of petroleum hydrocarbons with high molecular weight is usually limited during bioremediation. This is due to their low water solubility and strong sorption in micropores or organic matter. Nonetheless, this can be overcome by the addition of synthetic surfactants such as Triton X-102, Tween 80, and Genapol X150. These surfactants enhance the water solubility of hydrocarbons through the decrease of interfacial tension of hydrophobic and hydrophilic substances, thereby increasing their bioavailability (Johnsen, Wick, and Harms 2005; Zhang, Wang, and Yan 2011). However, these surfactants are not

without drawbacks as they can be toxic to macro- and microorganisms (Makkar and Rockne 2003). Similar to anthropogenic surfactants, microbial produced biosurfactants have been shown to improve the utilisation of hydrocarbons present in soil as shown in Figure 1-2 (Pacwa-Płociniczak et al. 2011).

Bacterial surfactants emulsify hydrocarbons by enhancing their water solubility, decreasing surface tension and increasing the displacement of oily substances from soil particles. They are diverse and, due to their low toxicity, environmentally friendly, and may perform well under extreme conditions. Microorganisms, especially bacteria, fungi and yeast, are known to be major producers of surfactants (Franzetti et al., 2010; Joshi and Desai 2010; Paulino et al. 2016; Borah and Yadav 2017). These microorganisms produce surfactants with different chemical structures including polysaccharides, fatty acids, glycolipids, peptides and proteins with hydrophobic and hydrophilic moieties that diminish surface and interfacial tensions between individual molecules (Mulligan 2005; Pacwa-Płociniczak et al. 2011). Degradation of petroleum hydrocarbons by surfactant-producing microorganisms is enhanced by two mechanisms which are increasing the substrate availability for microorganisms and interaction with cell surface, which increases the hydrophobicity of the surface that allows hydrophobic substrates to associate more easily with bacterial cells (Deziel et al. 1996; Nguyen et al. 2008; Cameotra and Singh 2008). Surfactants are classified into two classes: low molecular weight surfactants and high molecular weight surfactants/biopolymers.

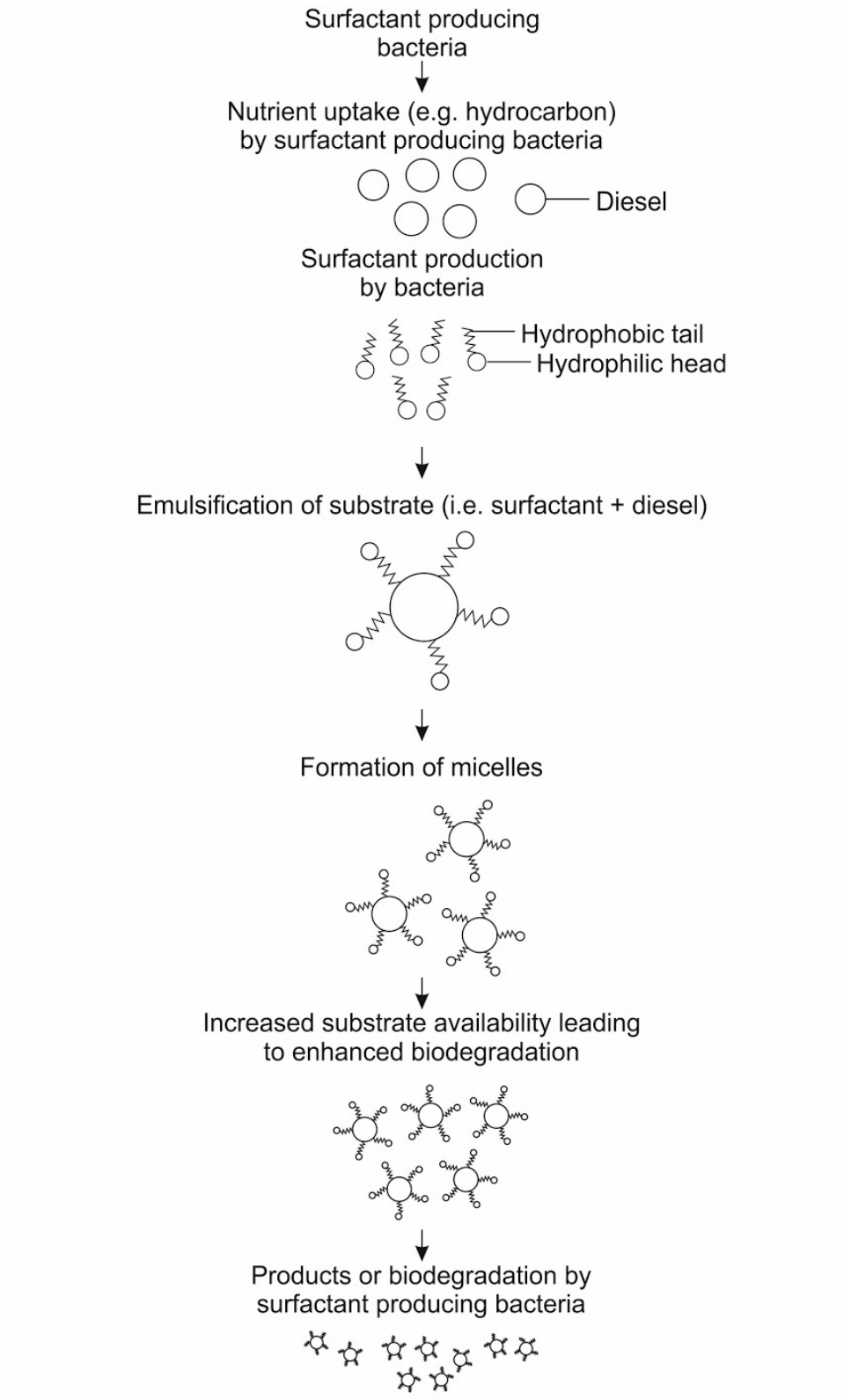


Figure 1-5. The mode of action of surfactants in the degradation of hydrocarbons. Hydrocarbon degrading bacteria facilitate the uptake of hydrocarbons by producing surfactants. The surfactants produced by hydrocarbon-degrading bacteria allows for the emulsification of hydrocarbons leading to enhanced bioavailability and degradation of hydrocarbons.

1.10 Low molecular weight surfactants

The low molecular weight surfactants are classified into five categories namely glycolipids, fatty acids/phospholipids, lipopeptides, polymeric and surfactant particles (Marcoux et al. 2000; Ron and Rosenberg 2002).

1.11 Glycolipids

Glycolipids are surfactants that have carbohydrates linked to long-chain aliphatic acids. They contain a hydrophobic fraction of one or more long chain aliphatic fatty acid in combination with different mono, di-, tri- and tetra-saccharides. Glycolipids are the most common and best studied surfactants as they have higher production yield than other surfactants (Kitamoto, Isoda, and Nakahara 2002; Müller and Hausmann 2011).

The biosynthesis of glycolipids involves the supply and linking of glycosyl and lipid precursors. The glycolysis and lipids precursors are linked through *O*-glycosidic, or ester bonds produced by glycosyltransferases or acyltransferases (Williams and Thorson, 2009; Rottig and Steinbuche, 2013). The glycosyltransferases catalyse the transfer of the sugar moiety from an activated glycosyl donor which in most cases is a sugar-nucleotide or a phosphate to a lipid acceptor by making glycosidic bonds between the hydroxyl groups of the acceptor and the anomeric carbon of the sugar donor. Acyltransferases aid the catalysis of the transfer of lipid moiety from an activated acyl donor such as acyl-CoA, to a glycosyl acceptor by producing an ester bond between the hydroxyl group of the acceptor and the acyl donor's carbonyl group (Rottig and Steinbuche, 2013). The commonly known glycolipids are described below.

- ***Rhamnolipids***

Rhamnolipids are well-known surfactants produced mainly by *Pseudomonas* spp. (Mnif and Ghribi 2015). In rhamnolipids, one or two rhamnose molecules are linked to one or two fatty acids, which can be a saturated or unsaturated alkyl acid. The fatty acid length varies from 8-14 carbon molecules. In *Pseudomonas* spp., e.g. *Pseudomonas aeruginosa*, plasmid

encoded genes, forming the *rhlABRI* gene cluster, are responsible for the production of rhamnolipids. The RhlA is responsible for the synthesis of the fatty acid dimer moiety of rhamnolipids, while rhlB and the rhlC are responsible for the transfer of dTDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) or a mono-rhamnolipid that was previously generated (Déziel et al. 2003). For the biosynthesis of rhamnolipids, the genes involved are plasmid encoded. The rhlA, B, R and I genes required to produce rhamnolipids are transcribed in the 5' -rhlABRI-3' direction (Ochsner et al., 1995).

Pseudomonas sp. MIS38 produces arthrofactin which is known to be twice as effective as surfactin produced by *Bacillus* sp. The arthrofactin synthetase gene cluster is formed by three genes known as arfA, arfB and arfC. These genes encode *ArfA*, *ArfB* and *ArfC* which assemble to form a unique structure (Roongsawang et al. 2003). *ArfA*, *ArfB* and *ArfC* contain two, four and five modules respectively. A module is a unit that catalyses the addition of a specific amino acid into the peptide product. The modules of a peptide synthetase arrangement is colinear with the amino acid sequence of the peptide. A module can be subdivided into different domains which are characterised by a set of short conserved sequence motifs. For every module, there is a condensation domain which is responsible for the formation of peptide bond between two consecutively bound amino acids, an adenylation domain which is responsible for the recognition of amino acids and adenylation at the expense of ATP and the thiolation domain which serves as an attachment site of 4-phosphopantetheine co-factor and a carrier of thioesterified amino acid intermediates. It is important to note that none of the modules possess the epimerization domain which converts amino acid residues from L to D form (Roongsawang et al. 2003).

Viscosin, a cyclic lipopeptide produced by *Pseudomonas fluorescens* SBW25, is synthesised by a non-ribosomal peptide synthetase (NRPS) encoded by three genes, namely viscA, viscB and viscC (Braun et al. 2001). The *viscA* gene is not clustered with *viscB* and *viscC* and is located at a different locus of the *Pseudomonas* genome with the distance of more than 1.5 MB (Roongsawang, Washio and Morikawa, 2007).

- ***Trehalose lipids***

Trehalose lipids contain the disaccharide trehalose linked at the C_6 to two β-hydroxy-branched fatty acids. Trehalose lipids are, for example, produced by *Mycobacteria*, *Norcadia*, *Rhodococcus* and *Corynebacteria.* Trehalose is synthesised by four possible pathways. The first is the TreYZ pathway, the second is the TreS pathway, and the third is the TreT is involved in the formation of trehalose by glycosyltransferase (Qu et al., 2004; Tsusaki et al, 1997; Maruta et al., 1995). The fourth pathway is the OtsAb pathway which is the best characterised one and the most important for the biosynthesis of trehalose (Murphy et al., 2005; Elbein et al., 2003). Trehalose lipids are constituents of the cell wall of *Rhodococcus* and are likely produced through the OtsAB pathway. In the OtsAB pathway, a trehalose-6-phosphate synthase (TPS, OtsA) catalyses the generation of trehalose-6-phosphate from a glucosyl nucleotide and glucose-6-phosphate. The second step involves a trehalose-6-phosphate (OtsB) catalysing the dephosphorylation to produce trehalose (Shimakata and Minatogawa, 2000; Minnikin et al., 2002). In *Rhodoccocus*, trehalose lipids are overproduced when grown on medium to long chain n-alkanes (Rapp and Gabriel-Jürgens, 2003).

- ***Sophorolipids***

Sophorolipids contain the disaccharide sophorose linked to a C_{16} or C_{18} fatty acid tail (Van Bogaert et al. 2007). Sophorolipids are produced by yeasts such as *Candida bombicola* and *Candida tropicalis* (Paulino et al. 2016). Even though there are no complete information available on the core biochemical pathway and the genes involved in the biosynthesis of sophorolipids, the hypothetical biochemical pathway is briefly explained: There are five genes directly involved in the synthesis of sophorolipids. These genes code for a cytochrome P450 monooxygenase, two glucosyltransferases, an acetyltransferase and a transporter. The genes are flanked by a putative alcohol dehydrogenase and a gene of unknown function at the 5’ and 3’ site respectively (Van Bogaert et al., 2013). Briefly, a hydroxylated fatty acid is glycosylated in an iterative manner which produces a non-

acetylated acidic sophorolipid which can further be replaced by one or two acetylation of the sophorose disaccharide and internal esterification. Although the sequence of the latter event is unclear, some enzymes responsible in the synthesis have not been identified (Ma et al., 2019).

1.12 Lipopeptides

Lipopeptides are surfactants composed of a peptide, that is linked to fatty acid. The peptides can either be linear or cyclic, and the fatty acids of varying length and structure. Generally, the fatty acids have chain lengths of C_{13} to C_{16} and may be branched. They are mainly produced by *Bacillus* spp. and are classified as surfactin, fengycin, iturin and lichenysin (Lang 2002; Nitschke and Pastore 2006; Mulligan 2009; Smyth et al. 2010). They are often employed in the biodegradation of hydrocarbons (Parthipan et al 2017). Lipopeptides are synthesised by non-ribosomal peptide synthetase (NRPS) via a thiotemplate process (Bushley and Turgeon, 2010). NRPS are multi-modular proteins that recognise, activate, alter and link the amino acids intermediate to the product peptide (Finking and Marahiel, 2004). Non-ribosomal peptides are biosynthesised through the function of the catalytic unit known as a module. Modules are important for the different reactions that leads to peptide transformation. A module is divided into many catalytic domains which are important for biochemical reaction. An NRPS module contains about 1000 amino acid residues and oversees one reaction cycle of selective substrate recognition and activation as an adenylate domain (A domain), tethering of a covalent intermediate as an enzyme -bound thioester i.e. Peptide carrier domain (PCP domain) and peptide bond formation which is the condensation domain (C domain) (Finking and Marahiel, 2004). The domains contained in the can be elongated by an E-domain and other substrate domains. The E domain is known as a domain for substrate epimerisation. The domains contained in the module can also be elongated by hydroxylation, methylation and heterocyclic ring formation which can be inserted at specific location into the module or as an independent catalytic unit. The thioesterase domain which is present in the last module ensures the cleavage of the

thioester bond between the nascent peptide and the last PCP-domain. The thioesterase is important for the cyclisation of the peptide (Ongena and Jacques, 2008).

- ***Surfactin***

Surfactin is a lipopeptide produced by *Bacillus subtilis* ATCC 21332. It belongs to the cyclic lipoheptapeptides containing β-hydroxyl fatty acids and seven D-/L-amino acid residues. The amino acid ring structure is linked to the fatty acid via a lactone linkage (Desai and Banat 1997; Ahimou et al. 2000). Surfactin produced by *Bacillus* spp. is catalysed by the NRPS surfactin synthetase complex. The surfactin synthetase complex is made up of four enzymatic subunits which are srfA, srfB, srfC and srfD. The *sfp* gene is also essential to produce surfactin. It is a gene encoding phosphopantethein transferase, which is necessary for the activation of the surfactin synthetase by posttranslational modifications (Steller et al. 2004).

- ***Fengycin***

Fengycin,also known as plipastatin, is a lipopeptide produced by many strains of *Bacillus* spp. Fengycin contains β-hydroxy fatty acid linked to a peptide moiety composed of ten amino acids in which eight are organised in a cyclic structure. Fengycin has antifungal activity against filamentous fungi (Akpa et al 2001). The synthetase operons are encoded by five genes from *fenA* to *fenE* (Wu et al, 2007). The *FenC* is responsible for the activation and assembling the second and last amino acids while *FenB* is responsible for the assemble of the last amino acid. *FenC* is the NRPS initiation module responsible for the synthesis of fengycin (Cheng et al. 2017).

- ***Iturin***

Iturin has a small molecular mass of 1.1 kDa. It is composed of a peptide moiety made up of seven amino acid residues and 11-12 carbon hydrophobic tail. Iturin A is the most common member of the iturin group. Other members of iturins are C, D, E, bacillopeptin and mycosubtilin. Iturins are of great importance in various industries due to their biological and

physical properties (Aranda et al.,2005; Tsuge et al.,2001). The synthesis of iturin occurs by a heterogeneous polyketide synthetase (PKS)-NRPS (Bland 1996). Members of the iturin group are cyclic octapeptide containing seven α-amino acids and a unique β-amino acid. Iturin A is composed of more than four open reading frames i.e. *ituD, ituA, ituB, and ituC* (Ongena and Jacques, 2008; Stein, 2005).

- ***Lichenysin***

Lichenysin is a cyclic lipopeptide produced by *Bacillus licheniformis*. It is synthesised non-ribosomally by a multienzyme peptide synthetase complex (Madslien et al. 2013). The lichenysin operon is made up of three peptide synthetase genes *licA, licB* and *licC* and they are transcribed in the same direction (Yakimov et al. 1998). Lichenysin is very stable, i.e. it possesses excellent stability under extreme temperature, pH and salt conditions. The lichenysin biosynthesis gene cluster is composed of four open reading frames i.e. *lchAA*, *lchAb*, *lchAC* and *lchA-TE* which encode the lichenysin synthetase LchAA, LchAB, LchAC and thioesterase LchA-TE. LchAA, LchAB, and LchAC are made up of three and one functional modules respectively (Nerurkar 2010(Nerurkar 2010; Yakimov, Fredrickson and Timms, 1996). Lichenysin has similar characteristics to surfactin (Nerurkar 2010).

1.13 Fatty acids/ phospholipids

These surfactants are produced by many bacteria and yeast during growth on n-alkanes to enhance the uptake of hydrocarbons. An example is *Acinetobacter* sp.1-N , which produces phospholipid ethanolamine-rich vesicles to form optically clear micro-emulsions of alkanes in water (Desai and Banat 1997).

1.14 Polymeric surfactants

Polymeric surfactants contain a polysaccharide backbone to which fatty acid side chains are covalently linked. Examples are emulsan, liposan, and alasan (also known as lipomannan). Alasan is a class of biosurfactant which aids the stabilization of hydrocarbon-in-water emulsions. It is produced by *Acinetobacter radiotolerens* KA53 (Toren et al. 2001). Liposan,

is an extracellular water-soluble emulsifier produced by *Candida lipolytica* and contains 83% carbohydrate and 17% protein. The carbohydrate portion is heteropolysaccharide made up of glucose, galactosamine and galacturonic acid (Ramana and Karanth, 1989). Liposan is known to emulsify edible oils by coating oil droplets to form emulsions (Cirigliano and Carman 1985). The genes responsible for the biosynthesis of polymeric surfactants in *Acinetobacter calcoaceticus* are organised in a cluster of about 60 kb. These genes were identified in a cosmid library used to complement non-surfactants producing mutants (Stark, 1996). The first gene involved in the synthesis of surfactants in *Acinetobacter calcoaceticus* is a homologue of genes that code for phosphoglucoisomerase (*pgi*) which carries out the bidirectional conversion of glucose-6-phosphate to fructose-6-phosphate. Other genes present in the cluster (*epsX* and *epsM*) indicate homology to the genes coding for GDP-mannose pyrophosphorylase and phosphomannose isomerase of enteric bacteria (Stark, 1996).

Alasan is a complex of ionic polysaccharide composed of apoalasan which is a covalently bound alanine and three proteins. It is released into the extracellular fluid during the stationary phase of the *Acinetobacter radiotolerens* KA53. The preparative SDS-PAGE of the alasan complex produces three proteins (AlnA, AlnB and AlnC). The AlnA is of 45kDa and all the emulsification activity occur here. The AlnA is encoded by the *alnA* gene (Toren et al. 2002). The AlnB has no emulsifying activity but is capable of stabilising oil-in-water emulsion produced by AlnA. *Acinetobacter radiotolerens* KA53 releases AlnA, AlnB and AlnC as a complex under stressed condition (Rosenberg et al. 2005).

Emulsan is produced by *Acinetobacter lwoffi* RAG1. It is secreted as a minicapsule on the cell surface and released into the medium as a protein-polysaccharide complex when the cells grow into stationary phase. The release occurs when an esterase is removed and an apoemulsan i.e. a polymer is formed. The apoemulsan is not capable of emulsifying non-polar, hydrophobic, aliphatic materials. For the biosynthesis emulsan, a 27 kb gene cluster known as wee encodes the genes *wza, wzb, wzc, wzx* and *wzy* (Nakar and Gutnick, 2003).

1.15 High molecular weight (HMW) surfactants/biopolymers

High molecular weight surfactants/biopolymers are generally known as polymeric surfactants. They contain either a mixture of polysaccharides, lipopolysaccharides, protein, and lipoproteins. They are similar to surfactants and are very effective in emulsifying hydrophobic substrates at low concentrations but less effective at reducing interfacial tensions (Inès and Dhouha 2015). They are also effective at solubilising poorly soluble substrates enhancing the ability for microbial hydrocarbon degradation (Uzoigwe et al. 2015).

1.16 Aims and Objectives of study

Epiphytic bacteria contain hydrocarbon degrading genes, but it is unclear if they gain fitness by being able to degrade hydrocarbons in the phyllosphere. This thesis addresses the hypothesis that surfactant production by epiphytic bacteria enhances hydrocarbon degradation in the terrestrial environment. The aims and objectives of this thesis were:

- To determine the ability of selected epiphytic bacteria to produce surfactants and to degrade hydrocarbons.

- To determine if the surfactants produced by the epiphytic bacteria facilitated their colonisation of the phyllosphere by using both surfactant-producing wild type and mutant non-surfactant-producing strains; and

- To test the impact of surfactants produced by the epiphytic bacteria in the degradation of hydrocarbons in soil microcosms by inoculating selected bacteria into soil microcosms containing petroleum and determining the degree to which the bacteria degraded the petroleum hydrocarbons.

In Chapter 2 the ability of the selected epiphytic bacteria to degrade hydrocarbons and produce surfactants was investigated. This chapter is published in FEMS microbiology letters.

Chapter 3 examined the roles of surfactants produced by epiphytic bacteria in the degradation of hydrocarbons. We also studied the ecophysiological functions of surfactants for bacteria in the phyllosphere. This chapter has been submitted for publication and a preprint is available at biorxiv (https://doi.org/10.1101/2020.10.27.358416).

In Chapter 4, bacteria were inoculated into soil microcosms containing petroleum amended sterile soil to determine the degree to which the bacteria degraded petroleum hydrocarbons under more natural environmental conditions. Residual oil was analysed using GC-FID.

Chapter 5 is a general discussion of the effects of surfactants on the degradation of hydrocarbons by epiphytic bacteria and on the ecophysiology of bacteria in the phyllosphere.

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2 Utilisation of hydrocarbons and production of surfactants by bacteria isolated from plant leaf surfaces.

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2.1 Abstract

Leaves are covered by a cuticle composed of long (C_{11}-C_{20}) and very-long chain hydrocarbons (>C20), alkanes, fatty acids, alcohols, aldehydes, ketones and esters. In addition to these aliphatics, cyclic hydrocarbons may be present. Leaves are colonised by a variety of so-called epiphytic bacteria, which may have adapted to be able to utilise cuticle hydrocarbons. We tested the ability of a wide range of phylogenetically different epiphytic bacteria to utilise and grow on diesel and petroleum benzine and show that out of the 21 strains tested, 9 had the ability to utilise diesel for growth. Only one strain was able to utilise petroleum benzine for growth. The ability to utilise hydrocarbons for growth correlated with the ability of the strains to produce biosurfactants and out the 21 tested strains, 12 produced surfactants. Showing that 75 % of the strains producing surfactants were able to degrade hydrocarbons. Our findings suggest that the ability to degrade hydrocarbons and to produce surfactants is highly prevalent in epiphytic bacteria. It is unclear if epiphytic bacteria utilise hydrocarbons originating from the cuticle of living leaves. The application of surfactant producing, hydrocarbon-utilising, epiphytic bacteria might serve as a method for hydrocarbons bioremediation.

2.2 Introduction

The bacterial habitat presented by the above ground organs of plants is referred to as the phyllosphere (Ruinen 1956). The phyllosphere is dominated by leaves, which are covered by a hydrophobic cuticle. The cuticle is a composite structure of cutin, a polymer consisting of crosslinked very long chain aliphatics, and different soluble aliphatics called cuticular waxes (Kolattukudy 1980; Müller and Riederer 2005). Cuticular waxes are either impregnated into the cutin matrix or overlayed on top of the matrix, i.e. intracuticular or epicuticular waxes, respectively (Buschhaus and Jetter 2011; Zeisler and Schreiber 2016). Intracuticular waxes serves the purpose of limiting non-stomatal water loss, while epicuticular waxes protects the leaf against UV-B radiation and biotic stresses (Barthlott and Neinhuis 1997; Buschhaus and Jetter 2011; Zeisler and Schreiber 2016). These waxes are mostly composed of very long chain alkanes (>C20), aldehydes, primary and secondary alcohols, ketones and alkyl esters (Jetter, Kunst and Samuels 2006).

Leaves are hosts to a diverse microbiota such as bacteria, fungi and oomycetes with bacteria being the most abundant reaching up to an average of 10^4 - 10^5 bacteria per mm^2 (Remus-Emsermann *et al.* 2014; Remus-Emsermann and Schlechter 2018). Previous studies have shown the presence of oil degrading bacteria on plant leaf surface and investigated the diversity of alkane degradation genes in leaf surface bacterial communities (Gandolfi *et al.* 2017). However, it is unclear if bacteria colonizing leaf surfaces gain fitness advantages by being able to degrade aliphatic compounds on plant leaves. The presence of hydrocarbons in the cuticle of leaves is a potential source of carbon and energy for leaf colonizing microorganisms especially bacteria which have been estimated to cumulate to 10^{26} bacteria globally residing on plant leaves (Vorholt 2012).

To enhance the degradation of hydrocarbons, bacteria can produce amphiphilic compounds known as surfactants (Oberbremer, Müller-Hurtig and Wagner 1990; Bautista *et al.* 2009). Surfactants reduce surface tension by accumulating at the interface of immiscible fluids increasing the surface area of insoluble compounds leading to increased bioavailability and

degradation of hydrocarbons. In that way, surfactants not only aid the bioavailability of hydrocarbons, but they also provide other fitness advantages to leaf colonising bacteria (Burch *et al.* 2014).

Previously, it was highlighted that some endophytic bacteria are able to degrade oil. This was proposed to have implications for plant-based bioremediation approaches (Phillips *et al.* 2008; Gkorezis *et al.* 2016; Pawlik *et al.* 2017). In the here presented study, we investigated the ability of 21 bacterial strains to degrade common hydrocarbons i.e. diesel and petroleum benzine. These strains represent the diversity of the leaf microbiota (Ito and Iizuka 1971; Rivas *et al.* 2004; Feil *et al.* 2005; Innerebner, Knief and Vorholt 2011; Remus-Emsermann *et al.* 2013, 2016; Bai *et al.* 2015). We determined the prevalence of the ability to degrade hydrocarbons and production of surfactants.

2.3 Materials and methods

2.3.1 Culture Media

Bacterial growth media used in this study: Lysogeny broth agar (LBA, Oxoid), lysogeny broth (Oxoid), nutrient agar (Oxoid), nutrient broth (Oxoid), R2A agar (Hi-media), R2A broth (0.5 g L^{-1} proteose peptone, 0.5 g L^{-1} glucose, 0.1 g L^{-1} $MgSO_4 \times 7H_2O$, 0.3 g L^{-1} $C_3H_3NaO_3$, 0.5 g L^{-1} yeast extract, 0.5 g L^{-1} casein acid hydrolysate, 0.5 g L^{-1} soluble starch, and 0.3 g L^{-1} K_2HPO_4), and half strength R2A broth (0.5 R2A). Bushnell Haas broth (BHB, 0.2 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2$, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} NH_4NO_3 and 0.05 g L^{-1} $FeCl_3$, pH 7.2). BHB was supplemented with diesel (commercial diesel, locally sourced), petroleum benzine (CAS 64742-49-0, Merck), or glucose, sucrose and/or succinate depending on strain preference (see Table 2-1). The diesel used in this study was previously analysed and consists mostly of C_{12}-C_{20} alkanes (Wante and Leung 2018). While alkanes and their branched derivatives in the range of C_9-C_{29} were detected, no evidence for fatty acid methylesters indicative for biodiesel could be detected. Aromatic components of the used diesel were not analysed, they usually contribute to ~25% of diesel and are mostly represented by cycloparaffins, alkylbenzenes and naphthalenes (Anonymous 1989a). A compositional

analysis of the petroleum benzine is not available, but usually consists of short chain aliphates, naphthenes, and small proportions of aromatics (Anonymous 1989b). Bacteria and respective media used to cultivate them are shown in Table 2-1. The EPA import permit number for the epiphytic bacteria was NOC 100168. *E. coli* DH5α was used as negative control for hydrocarbon degradation and surfactant production and *Pseudomonas syringae* B728a was used as positive control for surfactant production. All bacteria were cultivated at 30 °C except for *Escherichia coli* DH5α, which was grown at 37 °C.

Table 2-1 Bacteria, surfactant production time and culture media used in study.

Strain	Control carbon source	Routine media	Source
Acidovorax sp. Leaf 84	Glucose	NA	(Bai *et al.* 2015)
Aeromicrobium sp. Leaf 245	Glucose	NA	(Bai *et al.* 2015)
Agreia sp. Leaf 335	Glucose	NA	(Bai *et al.* 2015)
Arthrobacter sp. Leaf 145	Glucose	NA	(Bai *et al.* 2015)
Escherichia coli DH5α	Glucose	NA	(Taylor, Walker and McInnes 1993)
Methylobacterium radiotolerans 0-1	Sucrose/ succinate	R2A	(Ito and Iizuka 1971)
Methylobacterium sp. Leaf 85	Succinate	R2A	(Bai *et al.* 2015)
Methylobacterium sp. Leaf 92	Succinate	R2A	(Bai *et al.* 2015)
Methylophilus sp. Leaf 414	Methanol	0.5 R2A	(Bai *et al.* 2015)
Microbacterium sp. Leaf 320	Glucose	NA	(Bai *et al.* 2015)
Pantoea agglomerans 299R	Glucose	NA	(Remus-Emsermann *et al.* 2013)
Plantibacter sp. Leaf 1	Glucose	NA	(Bai *et al.* 2015)
Pseudomonas citronellolis P3B5	Glucose	NA	(Remus-Emsermann *et al.* 2016)
Pseudomonas syringae B728A	Glucose	NA	(Feil *et al.* 2005)
Rathayibacter sp. Leaf 296	Glucose	NA	(Bai *et al.* 2015)
Rhodococcus sp. Leaf 225	Glucose	R2A	(Bai *et al.* 2015)
Sphingomonas melonis Fr1	Sucrose/ succinate	R2A	(Innerebner, Knief and Vorholt 2011)
Sphingomonas phyllosphaerae FA2	Glucose/ sucrose	R2A	(Rivas *et al.* 2004)
Sphingomonas sp. Leaf 17	Glucose	R2A	(Bai *et al.* 2015)
Sphingomonas sp. Leaf 34	Succinate	R2A	(Bai *et al.* 2015)
Sphingomonas sp. Leaf 357	Succinate	R2A	(Bai *et al.* 2015)
Williamsia sp. Leaf 354	Glucose	R2A	(Bai *et al.* 2015)

2.3.2 Phylogenetic relationships of phyllosphere-colonising bacteria

A phylogenetic tree of the phyllosphere-colonising bacterial strains was constructed based on multilocus sequence analysis (MLSA) of six single-copy marker genes (*dnaG*, *infC*, *nusA*, *pyrG*, *rplA*, and *rpoB*). Marker genes were retrieved from the previously sequenced genomes using the AMPHORA2 pipeline (Wu and Scott 2012). Predicted amino acid sequences were concatenated and aligned using MUSCLE (Edgar 2004). Phylogenetic analysis and tree construction were performed in MEGA7 (Kumar, Stecher and Tamura 2016). The phylogeny model used was maximum-likelihood with 1000 bootstraps (Figure 2-1). The genome accession numbers for each bacterial strain can found in appendix 1.

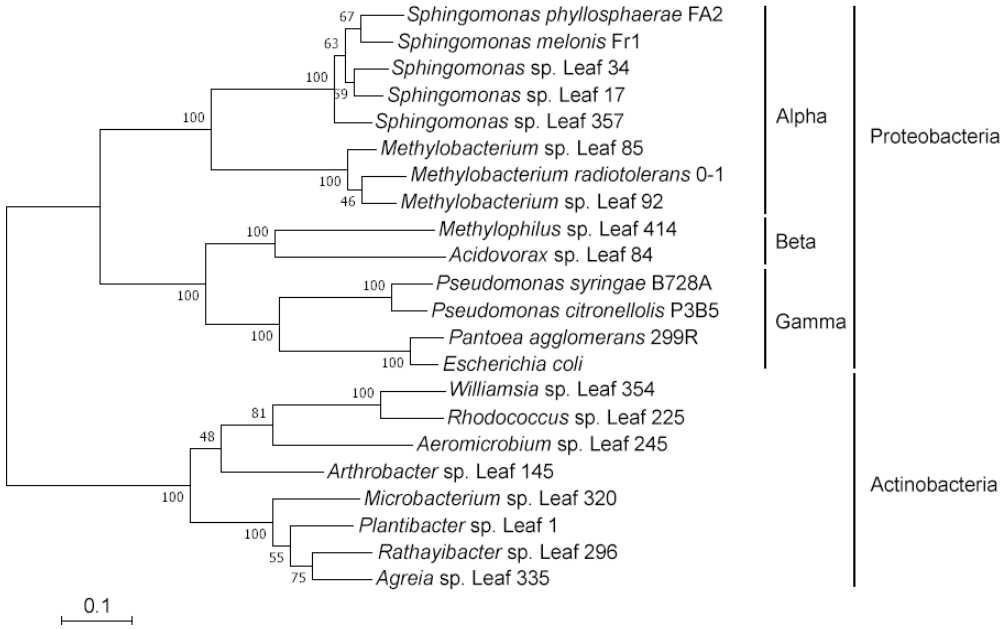

Figure 2-1 Maximum-likelihood phylogenetic tree of the bacteria employed in this study. The tree is based on multilocus sequence analysis. Six single-copy genes (dnaG, infC, nusA, pyrG, rplA, and rpoB) were concatenated and used to reconstruct the phylogenetic relationships of the bacteria. Nodes represent the confidence level of the phylogeny method with a bootstrap of 1000. The scale bar represents the number of substitutions per amino acid.

2.3.3 Hydrocarbon utilisation assay

To measure hydrocarbon utilisation by epiphytic bacteria, 250 mL conical flasks containing 50 mL of BHB supplemented with 1 % v/v hydrocarbon substrate (diesel or petroleum benzine) were used as sole carbon source. For positive controls BHB was supplemented with 1 % v/v methanol, or 1 % w/v glucose, sucrose or succinate as sole carbon source as indicated in

Table 2-1. BHB without additional carbon sources served as negative control. Media were inoculated with 0.5 mL of 100-times diluted overnight cultures (OD_{600} of the overnight cultures of most strains was between 0.9 and 1.4 save for *Methylophilus* sp. Leaf 414 and *E. coli* DH5α, which reached an optical density of 0.5 and 2, respectively). Cultures were incubated at 30 °C and 200 revolutions per minute (rpm) for up to 23 days. Cell density was measured by determining the optical density at 600 nm (OD_{600nm}) every 48 hours using a spectrophotometer (Biochrom WPA CO8000, Biowave). Non-inoculated BHB supplemented with diesel or petroleum benzine served as control for contaminations of the hydrocarbons and if the hydrocarbons alone change the medium's absorbance. All treatments were performed in three biological replicates.

2.3.4 Atomized oil assay

The atomized oil assay was performed as previously described (Burch *et al.* 2010). Briefly, freshly grown bacterial colonies were harvested from agar plates and suspended in 1 × phosphate buffer saline (PBS, 0.2 g L^{-1} NaCl, 1.44 g L^{-1} Na_2HPO_4 and 0.24 g L^{-1} KH_2PO_4). The OD_{600nm} was adjusted to 0.5 with PBS and 2 µL of the suspension were pipetted onto NA, LBA or R2A agar plates according to the strains' optimal growth conditions (Table 2-1). Plates were incubated at 30° C for up to 5 days, depending on the growth rate of the strains. A fine mist of light paraffin oil (BDH laboratories) was then applied onto the agar plates using an airbrush gun with an air pressure of 100-140 KPa. As a positive control, 2 µL of a 10 % v/v Tween-20 solution (Sigma-Aldrich) was pipetted onto LBA. Surfactant-producing bacteria exhibited visible halos where the oil droplets reflected light differently due to the presence of surfactants that changed the hydrophobicity of the agar medium (see Figure 2-3). Halos were visualized by using an indirect light source and photographs of halos were taking using a dark field illumination technique on a photo stage.

2.3.5 Drop collapse assay

The drop collapse assay was performed as described previously (Bodour and Miller-Maier 1998; Burch *et al.* 2010). Briefly, 2 µL of Magnatec 10W-40 oil (Castrol) were pipetted into

each well of a 96-well plate lid (Corning incorporated) and were allowed to equilibrate for 2 hours to ensure that each well was evenly coated. Bacterial overnight cultures were centrifuged at 2600 × *g* for 10 minutes. 5 µL of the culture supernatant were pipetted into the centre of an oil filled well. Drops were observed for up to 5 minutes and the time to collapse was determined. Drops that collapsed into the oil, i.e. decreased their contact angle, were positive for surfactant production while drops that remained intact and stayed on top of the oil were negative for surfactant production.

2.4 Results

2.4.1 Growth of epiphytic bacteria on hydrocarbons

Out of the 21 bacterial strains tested, 9 were found to grow on diesel, including the Alphaproteobacteria *Sphingomonas* sp. Leaf 17, *Sphingomonas* sp. Leaf 34, *Sphingomonas* sp. Leaf 357; the Gammaproteobacteria *P. citronellolis* P3B5 and *P. syringae* B728A; and the Actinobacteria *Aeromicrobium* sp. Leaf 245, *Arthrobacter* sp. Leaf 147, *Rhodococcus* sp. Leaf 225, and *Williamsia* sp. Leaf 354 (Figure 2-2, Table 2-2). Bacterial strains that were able to utilise diesel for growth could do so to high culture densities. Of the strains that utilised diesel, *Arthrobacter* sp. Leaf 147 was the strain with the lowest density and grew to an OD_{600nm} of 0.9 after 20 days (Figure 2-2 D). *Sphingomonas* sp. 357 was the strain that grew to the highest culture density and reached an OD_{600nm} of 1.95 after 20 days (Figure 2-2 T). None of the tested strains grew on BHB without a carbon source. On diesel, bacteria needed several days before an increase in optical density was detectable. None of the strains growing on diesel exhibited a clear exponential growth phase, and growth appeared linear, rather than exponential. This is likely due the low solubility of diesel in the aqueous medium, i.e. the access to the carbon source is limited by the surface of oil droplets in the solution, rather than the absolute amount of diesel present. On BHB media supplemented with control carbon sources (see Table 2-1), all cultures grew to saturation after 1 - 5 days depending on the strain (data not shown). *Sphingomonas* sp. Leaf 17 was the only strain that was able grow on petroleum benzine to an OD_{600nm} of 0.39 approximately double the optical density of the negative control (Figure 2-2

R). Non-inoculated flasks supplemented with diesel or petroleum benzine did not exhibit a noteworthy increase of optical density, i.e. the hydrocarbons did not form an emulsion in BHB that would change the turbidity of the broth. *E. coli* DH5α, the negative control, did not grow on diesel or petroleum benzine.

Table 2-2 Bacterial hydrocarbon degradation and surfactant production in the investigated strains

Strain	diesel degradation	petroleum benzine degradation	atomised oil assay	drop collapse assay
Acidovorax sp. Leaf 84	-	-	-	0/12
Aeromicrobium sp. Leaf 245	+	-	+	5/12
Agreia sp. Leaf 335	-	-	-	0/12
Arthrobacter sp. Leaf 145	+	-	+	9/12
Escherichia coli DH5α	-	-	-	0/12
Methylobacterium radiotolerans 0-1	-	-	+	8/12
Methylobacterium sp. Leaf 85	-	-	+	7/12
Methylobacterium sp. Leaf 92	-	-	+	8/12
Methylophilus sp. Leaf 414	-	-	-	0/12
Microbacterium sp. Leaf 320	-	-	-	0/12
Pantoea agglomerans 299R	-	-	-	0/12
Plantibacter sp. Leaf 1	-	-	-	0/12
Pseudomonas citronellolis P3B5	+	-	+	7/12
Pseudomonas syringae B728A	+	-	+	7/12
Rathayibacter sp. Leaf 296	-	-	-	0/12
Rhodococcus sp. Leaf 225	+	-	+	8/12
Sphingomonas melonis Fr1	-	-	-	0/12
Sphingomonas phyllosphaerae FA2	-	-	-	0/12
Sphingomonas sp. Leaf 17	+	+	-	9/12
Sphingomonas sp. Leaf 34	+	-	-	7/12
Sphingomonas sp. Leaf 357	+	-	-	8/12
Williamsia sp. Leaf 354	+	-	+	7/12

* number of collapsed oil drops after up to 10 minutes.

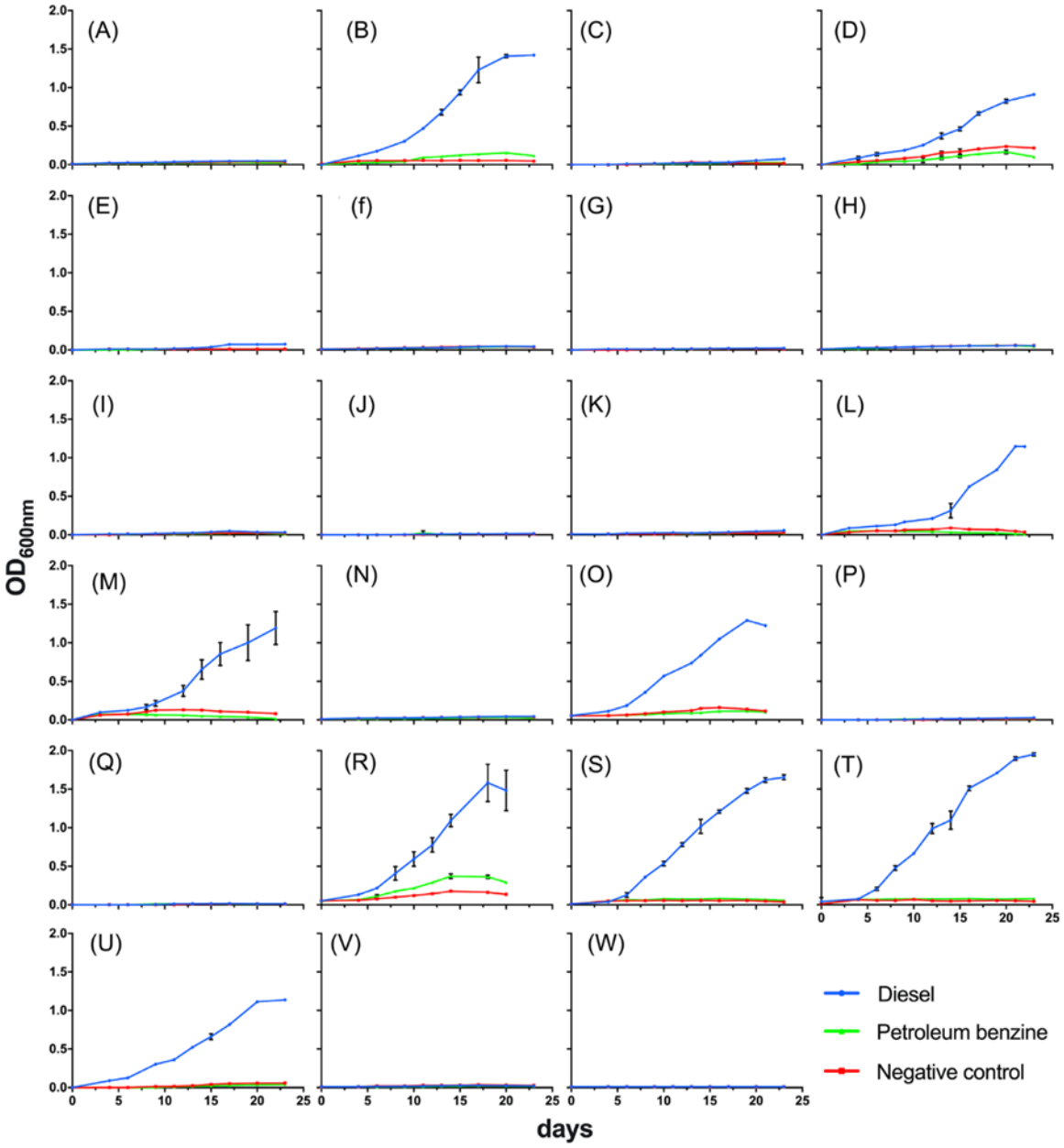

Figure 2-2 Growth of epiphytic bacteria on diesel, petroleum benzine, or non-supplemented Bushnell Haas broth.
(A) Acidovorax sp. Leaf 84, (B) Aeromicrobium sp. Leaf 245, (C) Agreia sp. Leaf 335, (D) Arthrobacter sp. Leaf 145, (E) Methylobacterium radiotolerans 0-1, (F) Methylobacterium sp. Leaf 85, (G) Methylobacterium sp. Leaf 92, (H) Methylophilus sp. Leaf 414, (I) Microbacterium sp. Leaf 320, (J) Pantoea agglomerans 299R, (K) Plantibacter sp. Leaf 1, (L) Pseudomonas citronellolis P3B5, (M) Pseudomonas. syringae B728A, (N) Rathayibacter sp. Leaf 296, (O) Rhodococcus sp. Leaf 225, (P) Sphingomonas melonis Fr1, (Q) S. phyllosphaerae FA2, (R) Sphingomonas sp. Leaf 17, (S) Sphingomonas sp. Leaf 34, (T) Sphingomonas sp. Leaf 357, (U) Williamsia sp. Leaf 354, (V) Escherichia coli DH5α, and (W) non-inoculated broth

2.4.2 Bacterial surfactant production

The atomized oil assay was used as initial screen to determine the production of surfactants by epiphytic bacteria. In this assay, the presence of amphiphilic compounds changes the conformation and reflection of oil droplets that are sprayed onto agar plates resulting in halos around control surfactants such as Tween-20 (Figure 2-3W) or bacterial colonies. When bacterial colonies were sprayed with a fine mist of light paraffin oil, halos were observed around 9 strains including the Alphaproteobacteria *Methylobacterium radiotolerans* 0-1, *Methylobacterium* sp. Leaf 85, *Methylobacterium* sp. Leaf 92; the Gammaproteobacteria *P. citronellolis* P3B5 and *P. syringae* B728A; and the Actinobacteria *Aeromicrobium* sp. Leaf 245, *Arthrobacter* sp. Leaf 147, *Rhodococcus* sp. Leaf 225, and *Williamsia* sp. Leaf 354 (Figure 2-3B, D, E, F, G, L, M, O, U; Table 2-2) indicating the presence of surfactants. All other strains did not exhibit halos, indicating a lack of surfactant production. The negative control *E. coli* DH5α did not produce a halo, while the commercial surfactant Tween 20 produced a halo.

The drop collapse assay determines the presence of compounds, such as surfactants, reducing the interfacial tension between engine oil and an aqueous solution, resulting in the aqueous solution to collapse into the oil. In the drop collapse assay, the culture supernatant of 12 epiphytic bacteria collapsed into oil, indicating that the strains were producing surfactants after growth in liquid media (Figure 2-4B, D, E, F, G, L, M, O, R, S, T, U; Table 2-2). This includes all nine strains that were found to produce surfactants in the atomised oil assay and the additional three strains *Sphingomonas* sp. Leaf 17, *Sphingomonas* sp. Leaf 34, and *Sphingomonas* sp. Leaf 357. For the strains negative for surfactant production, the supernatant did not collapse into oil and remained on top of the oil.

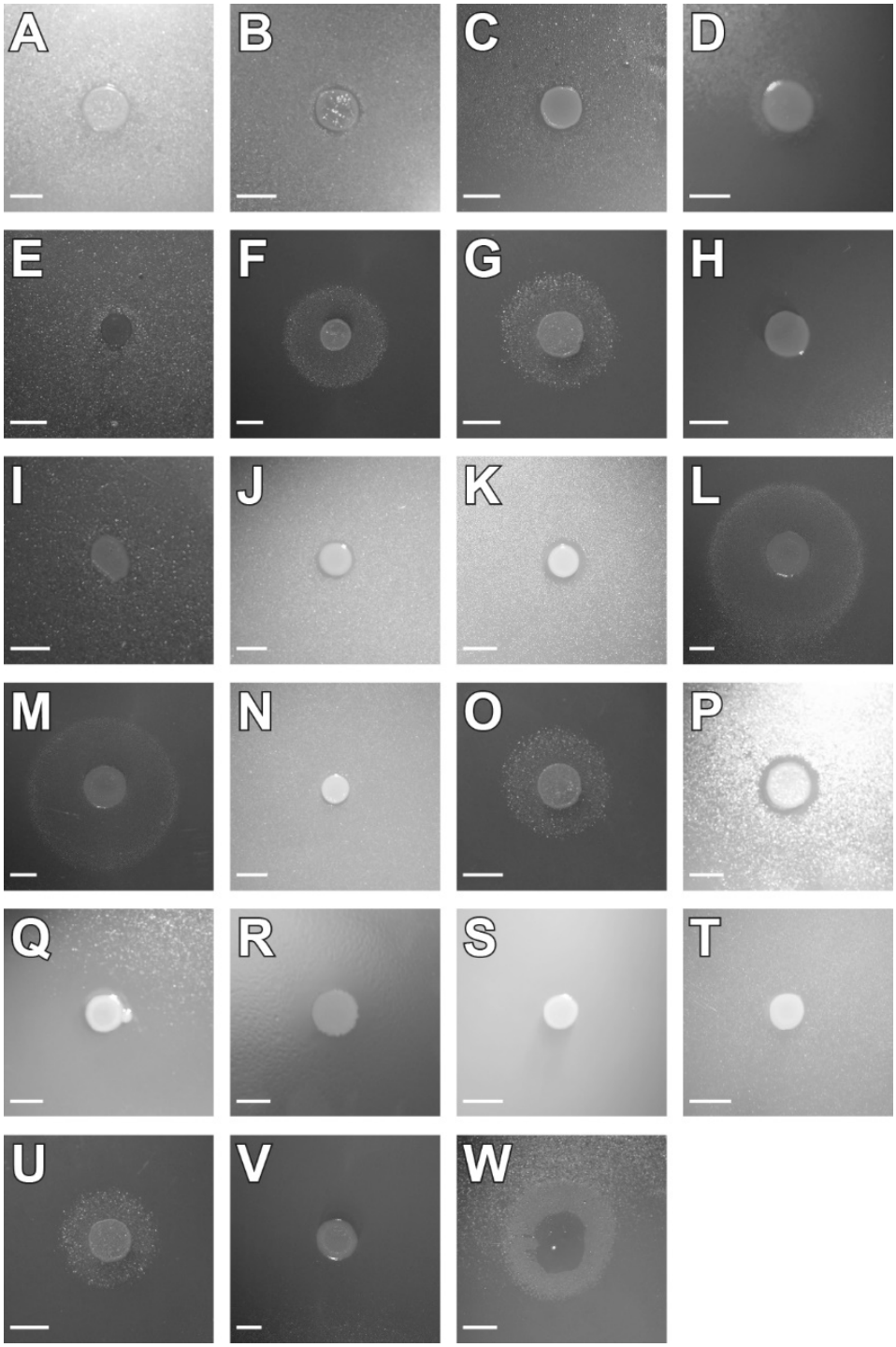

Figure 2-3 Atomized oil assay to screen for surfactant production.
(A) Acidovorax sp. Leaf 84, (B) Aeromicrobium sp. Leaf 245, (C) Agreia sp. Leaf 335, (D) Arthrobacter sp. Leaf 145, (E) Methylobacterium radiotolerans 0-1, (F) Methylobacterium sp. Leaf 85, (G) Methylobacterium sp. Leaf 92, (H) Methylophilus sp. Leaf 414, (I) Microbacterium sp. Leaf 320, (J) Pantoea agglomerans 299R, (K) Plantibacter sp. Leaf 1, (L) Pseudomonas citronellolis P3B5, (M) Pseudomonas. syringae B728A, (N) Rathayibacter sp. Leaf 296, (O) Rhodococcus sp. Leaf 225, (P) Sphingomonas melonis Fr1, (Q) S. phyllosphaerae FA2, (R) Sphingomonas sp. Leaf 17, (S) Sphingomonas sp. Leaf 34, (T) Sphingomonas sp. Leaf 357, (U) Williamsia sp. Leaf 354, (V) Escherichia coli DH5α, and (W) Tween-20. Scale bars represent 5 mm.

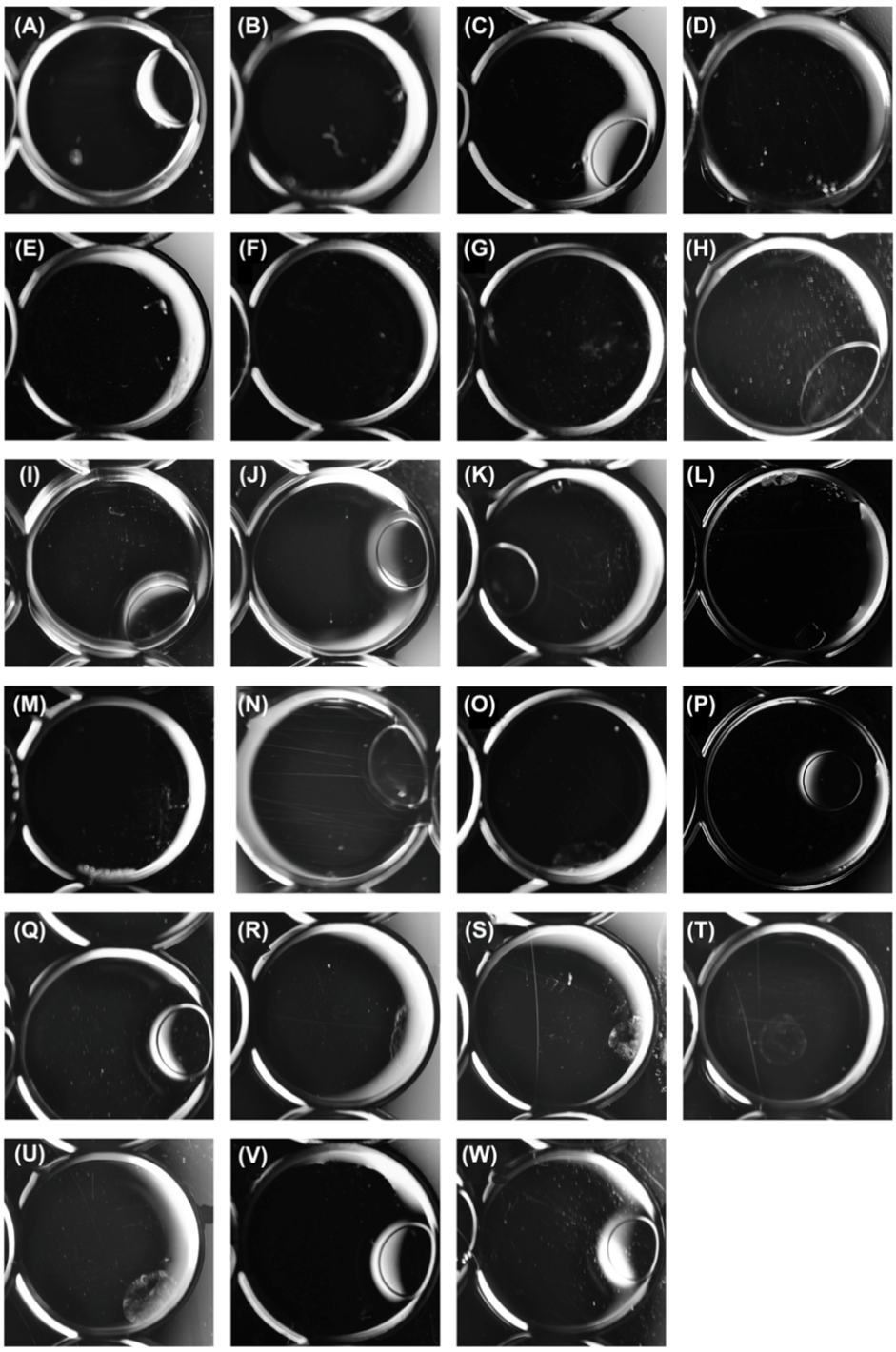

Figure 2-4 Drop collapse assay.
Negative results show beaded droplets of culture supernatant on top of oil, positive results do not exhibit beaded droplets, instead the drops appear collapsed. (A) Acidovorax sp. Leaf 84, (B) Aeromicrobium sp. Leaf 245, (C) Agreia sp. Leaf 335, (D) Arthrobacter sp. Leaf 145, (E) Methylobacterium radiotolerans 0-1, (F) Methylobacterium sp. Leaf 85, (G) Methylobacterium sp. Leaf 92, (H) Methylophilus sp. Leaf 414, (I) Microbacterium sp. Leaf 320, (J) Pantoea agglomerans 299R, (K) Plantibacter sp. Leaf 1, (L) Pseudomonas citronellolis P3B5, (M) P. syringae B728A, (N) Rathayibacter sp. Leaf 296, (O) Rhodococcus sp. Leaf 225, (P) Sphingomonas melonis Fr1, (Q) S. phyllosphaerae FA2, (R) Sphingomonas sp. Leaf 17, (S) Sphingomonas sp. Leaf 34, (T) Sphingomonas sp. Leaf 357, (U) Williamsia sp. Leaf 354, (V) Escherichia coli DH5α, and (W) Water.

2.5 Discussion

Previous studies indicate that plant leaves are hosts to hydrocarbon-degrading bacteria based on selective media and sequencing data (Al-Awadhi *et al.* 2012; Gandolfi *et al.* 2017). The here-presented results provide evidence that members of the epiphytic microbiota contain high relative proportions of hydrocarbon degrading bacteria that are able to degrade environmental hydrocarbons. The here-investigated 21 bacterial strains from the phyllosphere were tested for their ability to utilize diesel and petroleum benzine and exploit them for growth. Furthermore, it was tested if the strains were able to produce surfactants which may enhance the bioavailability of hydrocarbons. The strains were originally isolated on media not selecting for the ability to degrade hydrocarbons (Ito and Iizuka 1971; Rivas *et al.* 2004; Feil *et al.* 2005; Innerebner, Knief and Vorholt 2011; Remus-Emsermann *et al.* 2013; Bai *et al.* 2015; Gekenidis *et al.* 2017). Therefore, the here tested randomly chosen leaf colonising bacteria suggest that as many as 40 % of leaf colonising strains might be able to utilise diesel as a sole carbon source. Bacteria colonising the phyllosphere have previously been shown to degrade hydrocarbons and it has been suggested that they thereby facilitate hydrocarbon removal from the environments (Al-Awadhi *et al.* 2012; Stevens *et al.* 2017). Other bacterial genera that have been previously found to be able to degrade hydrocarbons include *Arthrobacter* (Efroymson and Alexander 1991), *Acinetobacter* (Mishra, Sarma and Lal 2004), *Methylobacterium* (Salam, Obayori and Raji 2015), *Pantoea* (Yousaf *et al.* 2010), *Pseudomonas* (Hasanuzzaman *et al.* 2007), *Sphingomonas* (Jones *et al.* 1983) and *Rhodococcus* (Huang *et al.* 2008). These genera are also found on leaves (Vorholt 2012; Bai *et al.* 2015). The role of surfactants in the bacterial bioremediation of hydrocarbons has been well studied (de Cássia F S Silva *et al.* 2014; Patel *et al.* 2019). Pseudomonads have been studied particularly well and many strains have been found to be able to utilise hydrocarbons. In addition it was shown that Pseudomonads are able to produce biosurfactants that increased the rates of remediation of hydrocarbons (Foght and Westlake 1988; Grimberg, Stringfellow and Aitken 1996; Beal and Betts 2000; Kumari, Singh and Singh 2012).

Previous studies have demonstrated that the production of surfactants helps the bean pathogen *Pseudomonas syringae* B728a to survive in the unfavourable environmental conditions of the phyllosphere (Lindow and Brandl 2003; Burch *et al.* 2014). Surfactants break the surface tension of water and lower the contact angle of water droplets on leaves (Knoll and Schreiber 2000). This increases the area that water droplets cover on leaves and increases the ability of bacteria to move on leaves as well as of nutrients to diffuse towards bacteria (Schreiber *et al.* 2005). Furthermore, bacterial surfactant production has also been shown to increase the wettability and permeability of leaf cuticles, which results in higher nutrient diffusion from the apoplast to the leaf surface. The production of surfactants by the here studied bacteria strains was assessed using two complementary assays (Bodour and Miller-Maier 1998; Burch *et al.* 2010). The assay revealed that about >50 % of the tested bacteria produced surfactants. Nine epiphytic bacteria, including the surfactant-producing bacterium *P. syringae* B728A (Burch *et al.* 2010; Hockett, Burch and Lindow 2013), produced halos in the atomized oil assay (Figure 2-3, Table 2-2). The drop collapse assay indicated the presence of surfactants in an additional 3 strains, i.e., in a total of 12 of the tested bacteria (Figure 2-4, Table 2-2). The drop collapse assay was therefore more sensitive to the detection of surfactants, or some bacteria were not forming surfactants on agar media. A high prevalence of surfactant producers has previously been shown in epiphytic bacterial communities (Burch *et al.* 2016). In a targeted isolation screen, Burch *et al.* (2016) found that the genera *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Erwinia*, *Exiguobacterium*, *Paenibacillus*, *Rhodanobacter*, and *Sphingobacterium* were surfactant producing isolates from the phyllosphere of different lettuce cultivars and spinach. Others were able to isolate surfactant-producing *Pseudomonas* from leaf surfaces of *Hedera helix* (Schreiber *et al.* 2005). Here, we expand this list with several strains from genera that were previously unknown to be surfactant producing epiphytes, including *Aeromonas*, *Arthrobacter*, *Methylobacteria*, *Sphingomonas*, *Rhodococcus*, and *Williamsia*. We noted that the atomised oil assay was not able to report on surfactant production of all strains tested in our screen. This may have previously resulted in

false negatives and an underestimate of surfactant producing bacteria colonising leaf surfaces.

All bacterial strains that were able to degrade hydrocarbons also produced surfactants and almost all bacteria that produced surfactants were also able to degrade hydrocarbons. The only bacteria that were not able to degrade hydrocarbons but produced surfactants were *Methylobacterium radiotolerans* 0-1, *Methylobacterium* sp. Leaf 85, *Methylobacterium* sp. Leaf 92. Potentially, the production of surfactants is important for their motility on leaves (Hockett, Burch and Lindow 2013) or surfactant production increases their access to nutrients by increasing the wettability and permeability of the leaf (Bunster, Fokkema and Schippers 1989; Schreiber *et al.* 2005). As mentioned above, other strains of this genus have previously been shown to be able to degrade hydrocarbons (Salam, Obayori and Raji 2015). It is unclear why these strains are not able to degrade hydrocarbons, however, one of the most common genes involved in alkane degradation *alkB* is not found in either *Methylobacterium* sp. leaf 85 or *Methylobacterium* sp. leaf 92. Potentially, BHB supplemented with diesel in not permissive to growth of *Methylobacteria*, however, all three strains were able to grow in BHB supplemented with sucrose and/or succinate. Bacterial surfactants have previously been well studied in the context of bioremediation of hydrocarbons (Banat *et al.* 2010; Rosenberg and Ron 2013; de Cássia F S Silva *et al.* 2014; Ron and Rosenberg 2014; Patel *et al.* 2019). Surfactants increase the bioavailability of hydrophobic substrates by reducing surface tension and increasing surface area of oil droplets. Therefore, the correlation between bacterial strains that degrade hydrocarbons and their ability to produce surfactants is not unexpected.

The here presented results show the prevalence of functional hydrocarbon utilising and surfactant producing bacterial species contributing to the plant leaf surface microbiota. This supports previous studies investigating the prevalence of the alkane degradation gene *alkB* on plant leaves of urban trees (Gandolfi *et al.* 2017). Our findings have implications for future bioremediation approaches.

2.6 Conclusion

Hydrocarbon utilising and surfactant producing bacteria are prevalent on leaf surfaces, this indicates that this functional trait might advantageous in the phyllosphere. Almost all species that were producing surfactants were also able to degrade diesel. If bacteria make use of their ability to degrade hydrocarbons and take advantage of the abundant very long chain aliphates that constitute the cuticular waxes and the cuticle itself is unclear and will be the subject of future studies. A thorough characterisation of the produced surfactants and analysis of oil degradation will be subject of future studies.

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3 Characterisation of the biosurfactants from phyllosphere colonising *Pseudomonads* and their effect on plant colonisation and diesel degradation

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3.1 Abstract

Biosurfactant production is a common trait in leaf surface colonising bacteria that has been associated with increased survival and movement on leaves. At the same time, the ability to degrade aliphatics is common in biosurfactant-producing leaf colonisers. Pseudomonads are common leaf colonisers and have been recognised for their ability to produce biosurfactants and degrade aliphatic compounds. In this study, we have investigated the role of biosurfactants in four non-plant pathogenic *Pseudomonas* strains by performing a series of experiments to characterise the surfactant properties, and their role during leaf colonisation and diesel degradation. The produced biosurfactants were identified using mass-spectrometry. Two strains produced viscosin-like biosurfactants and the other two produced Massetolide A-like biosurfactants which aligned with the phylogenetic relatedness between the strains. To further investigate the role of surfactant production, random Tn*5* transposon mutagenesis was performed to generate knockout mutants. The knockout mutants were compared to their respective wild types in their ability to colonise gnotobiotic *Arabidopsis thaliana* and to degrade diesel or dodecane. It was not possible to detect negative effects

during plant colonisation in direct competition or individual colonisation experiments. When grown on diesel, knockout mutants grew significantly slower compared to their respective wild types. When grown on dodecane, knockout mutants were less impacted compared to growth on diesel. By adding isolated wild type biosurfactants it was possible to complement the growth of the knockout mutants.

3.2 Introduction

The leaf cuticle is a hydrophobic barrier which consists of cutin, a polymer of very long chain aliphatics, interspersed and overlaid by very long chain monomeric aliphatics, cuticular waxes (Kolattukudy, 1980; Zeisler-Diehl et al., 2018). The cuticle reduces water loss, provides protection against UV radiation, and is the primary interface for plant microorganism and insect interactions (Riederer & Schreiber, 2001; Serrano et al., 2014; Yeats et al., 2012). The cutin is a biopolymer which consists mainly of ω −and midchain hydroxy and epoxy fatty acids C_{16}-C_{18} as well as glycerol (Graça, 2002; Pollard et al., 2008; Wattendorff & Holloway, 1980). The cutin forms the structural backbone of the cuticle as it is known to prevent mechanical damage. The cuticular waxes are the second major component of the leaf cuticle mostly consisting of alkanes, alcohols, acids, and aldehydes of chain lengths between C_{16} - C_{32}. Cuticular waxes may also include secondary metabolites such as flavonoids, triterpenoids and phenylpropanoids (Jeffree, 2006). Cuticular waxes can be separated into two distinct waxes. The intracuticular wax within the cutin polymer is clearly distinct from the epicuticular wax which is on the outer surface of the cutin polymer (Buschhaus & Jetter, 2011; Samuels et al., 2008). These differences thus affect the physical properties of the plant surfaces. The composition of the cuticular waxes is dependent on plant species and environmental conditions (Jetter et al., 2006; Shepherd & Wynne Griffiths, 2006). Wax monomers are very energy rich and a potential source of energy and carbon if they are bioavailable. However, it is still unclear if bacteria are able to utilise these aliphatic compounds constituting the cuticle of living leaves as a source of carbon and if surfactants would facilitate the utilisation.

Leaves are home to manifold bacteria and can be covered by up to 5% bacterial biomass (Remus-Emsermann et al., 2014; Schlechter et al., 2019). Many leaf surface colonising genera were previously shown to degrade hydrocarbons, e.g. *Rhodococcus* spp., *Sphingomonas* spp., *Pantoea* spp., *Methylobacterium* spp., and Pseudomonads (Kertesz & Kawasaki, 2010; Oso et al., 2019; Pizzolante et al., 2018; Salam et al., 2015). Pseudomonads are common leaf colonisers and have many different ecological roles, e.g. many *Pseudomonas syringae* strains can be bonafide and host specific pathogens (Xin et al., 2018) while others may act as antagonists against agents of plant disease (Cabrefiga et al., 2007; Zengerer et al., 2018) or have unknown, tritagonistic (Freimoser et al., 2016), functions in the microbiota (Remus-Emsermann et al., 2016; Schmid et al., 2018). Pseudomonads have the ability to produce so-called biosurfactants in common (D'aes et al., 2010). Biosurfactants are biologically produced amphiphilic molecules consisting of a hydrophilic head group and a hydrophobic moiety.

Leaf colonising Pseudomonads produce cyclic peptide biosurfactants (D'aes et al., 2010). Their ecophysiological role is not always clear, but it has been shown that Pseudomonads may gain different fitness advantages by producing surfactants including increasing survival during fluctuating humidity conditions on leaves (Burch et al., 2014) and by increasing local water availability due to the hygroscopic nature of their surfactants (Hernandez & Lindow, 2019). On agar plates it has been shown that biosurfactants increase surface mobility by swarming and it has been assumed that they may serve similar functions on leaves (Lindow & Brandl, 2003).

Originally, the work described in this chapter was aiming at generating surfactant mutants from *Pseudomonas citronellolis* P3B5, which was part of the survey described in chapter 2, using random transposon mutagenesis. Thousands of mutants were produced; however, no surfactant knockout mutants could be identified. Details can be found in appendix 2. Hence, mutants that were available elsewhere were used for the work further described in chapter 3. In this study, we characterised the physiological effect of biosurfactants in four different Pseudomonads that were isolated from leaves of spinach (*Pseudomonas* sp. FF1) or

Romaine lettuce (*Pseudomonas* spp. FF2, FF3, and FF4) respectively. Their biosurfactants were characterised using mass spectrometry and their physical properties were analysed. Furthermore, we investigated the ecophysiological functions of the biosurfactants for the bacteria. To that end, random insertion libraries were produced and biosurfactant knockout mutants identified. The knockout mutants were characterised in a series of experiments that investigated fitness changes *in vitro* and *in planta*.

3.3 Material and Methods

3.3.1 Bacterial strains used in this study.

Bacteria used in this study were *Pseudomonas* sp. FF1 (*P*FF1), *Pseudomonas* sp. FF2 (*P*FF2), *Pseudomonas* sp. FF3 (*P*FF3), *Pseudomonas* sp. FF4 (*P*FF4) (Burch et al., 2011); All Pseudomonads were kind gifts of Adrien Burch and Steven Lindow (UC Berkeley)) and *E. coli* Stellar (Lucigen) was used for cloning. *P*FF1 was isolated from spinach, *P*FF2, *P*FF3, and *P*FF4 were isolated from Romaine lettuce. Pseudomonads were routinely grown in liquid King's B (KB, 20 g proteose peptone, 1.15 g K_2HPO_4, 1.5 g $Mg[SO_4]*7H_2O$. 10 g glycerol per liter, pH 7; for agar medium KBA, add 15 g agar per liter) or Lysogeny Broth (LB, 5 g yeast extract, 10 g tryptone, 10 g NaCl per liter, pH 7; for agar medium add 15 g agar per liter). *E. coli* was routinely grown in LB and on LBA. For *in planta* competition experiments, spontaneous streptomycin resistant mutants of the wild type Pseudomonads were selected (Newcombe & Hawirko, 1949). Where appropriate, the media were supplemented with kanamycin (50 µg ml^{-1}) or streptomycin (50 µg ml^{-1}).

3.3.2 16S rRNA gene sequencing

To determine the phylogeny of the strains, their 16S rRNA genes were amplified from genomic DNA that was extracted using the NucleoSpin® Microbial DNA Kit (Macherey Nagel) following the manufacturer's recommendations. A PCR using KAPA2G Fast 2x Ready Mix with Dye (Kapa) was performed using the manufacturer's recommendation, 1 µL of genomic and 16S rRNA gene targeting primers SLK8-F 5'-

AGAGTTTGATCATGGCTCAGAT-3' and SRK1506-R 5'-TACCTTGTTACGACTTCACCCC-3'. Resulting ~1.5 Kbp fragments were sequenced (Eurofins Genomic) and then curated and assembled using Geneious prime (Geneious). The assembled fragments were uploaded to ezbiocloud (Yoon et al., 2017) and the 30 best matches of organisms that were validly named were recovered for each of the four strains. Additional *Pseudomonas* 16S sequences and outgroup sequences were recovered from the silva database (Glöckner et al., 2017). All sequences were compiled into a fasta file and aligned and visualised using the FastME/OneClick option of ngphylogeny.fr (Lemoine et al., 2019). The resulting tree was imported into iTol, edited for publication and then exported (Letunic & Bork, 2019).

3.3.3 Preparation of electrocompetent Pseudomonads

Electrocompetent Pseudomonads were produced as explained elsewhere (Artiguenave et al., 1997). Briefly, bacteria were grown overnight in 6 ml KB in a shaking incubator at 25 °C. Three ml of the overnight culture were then used to inoculate 100 ml KB that were incubated at 25 °C in a shaking incubator until the culture reached mid-exponential growth phase OD_{600nm} of approximately 0.6. The culture was then split in 50 ml aliquots and cooled on ice for 30 minutes. Bacteria were then harvested by centrifugation at 6000 *g* and 4 °C for 10 minutes. The supernatant was discarded and the aliquots were washed twice with 50 ml ice-cold sterile water. Then they were washed in 25 ml ice-cold water and the aliquots were combined again. After a final centrifugation, the cell pellet was resuspended in 250 µl sterile 10% glycerol and distributed in 50 µl aliquots that were stored at -80 °C.

3.3.4 Random transposon mutagenesis

Random knockout mutants were produced using the EZ::Tn5^{Tm} <KAN-2> Tnp Transposome^{Tm} kit (Epicentre), which confirms a kanamycin resistance marker, following the manufacturers recommendations. In brief, 50 µl electrocompetent Pseudomonads were thawed on ice and 1 µl Tn*5*-transposome and 1 µl endonuclease inhibitor were mixed with the cells. The mix was incubated for 5 minutes on ice before the cells were pipetted into a pre-chilled 0.1 cm gap electroporation cuvette. A gene pulser (Bio-Rad) was used to pulse

the cells (2.5 kV, 200 Ω, 25 µF). Immediately after that, 1 ml SOC (SOB: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl per liter, pH 7. SOC: SOB supplemented with 5 ml 2 M $MgCl_2$ and 20 ml 1 M glucose) was added and the cells were incubated for 1 hour at 30° C and 150 rpm. Transposon insertion mutants were selected on minimal medium agar plates (15 ml glycerol, 5 g L-glutamine, 1.5 g K_2HPO_4, 1.15 g $MgSO_4 \times 7H_2O$, 15 g agar per liter, pH 7) supplemented with kanamycin. Minimal medium was used to prevent the growth of auxotrophic mutants. Transposon mutants could be detected after 2 days.

To determine the site of transposon integration, genomic DNA of knockout mutants was isolated using the ISOLATE II kit (Bioline). Genomic DNA was cut using KpnI (New England Biolabs) or EcoRI and ligated into similarly digested and dephosphorylated vector pUC19 (New England Biolabs) using T4-ligase (New England Biolabs) following the recommendations of the manufacturer. 5 µl per ligation mix were transformed into chemical competent *E. coli* Stellar using the manufacturers recommendations. Clones harboring plasmids containing the transposon were selected on LB supplemented with kanamycin. Inserts of the plasmids were sequenced using the transposon specific primer kan2_RP-1 (5'-gcaatgtaacatcagagattttgag-3'). Sequencing results were compared to the NCBI database using NCBI BLAST restricted to the genus *Pseudomonas* (Altschul et al., 1990).

3.3.5 Screens for surfactant production

To screen for surfactant production, the atomised oil assay was performed (Burch et al., 2010). To that end, agar plates containing transposon mutants were sprayed with hydrophobic dodecan using an airbrush. Bacterial colonies that produced surfactants resulted in a halo around the colony where the surfactant in the agar changes the surface angle of oil droplets on the surface. Colonies that lacked this characteristic halo were further characterised. Presumptive surfactant mutants were tested in the drop collapse assay as described previously (Oso et al., 2019). Briefly, 2 µl of Magnatec 10W-40 oil (Castrol) were pipetted into each well of a 96-well plate lid (Corning incorporated) and were allowed to equilibrate for 2 hours to ensure that each well was evenly coated. Bacterial overnight

cultures were centrifuged at 2600 × *g* for 10 minutes. Five µL of the culture supernatant was pipetted into the centre of an oil filled well. Drops that collapsed into the oil, i.e. decreased their contact angle, were positive for surfactant production while drops that remained intact and stayed on top of the oil were negative for surfactant production. All experiments were performed in at least 8 biological replicates. To determine the doubling times of the wild type and surfactant knock out mutant strains, the exponential growth phase of every culture was used. To that end, the exponential part of the growth curve was log transformed and the slope was determined using a linear regression (Graph Prism). To determine the doubling time, the growth rate was calculated first. $growth\ rate\ N(t) = N(0)e^{gr*t}$. N(0)= number of cells at time 0; gr = growth rate; t = time. The growth rate was then used to calculate the doubling time: $\text{Doubling time} = \frac{\ln(2)}{growth\ rate}$.

3.3.6 Extraction of surfactants

Bacterial strains were grown as crude streaks on five separate KBA plates for 48 hours at 25 °C. Afterwards, bacterial biomass was harvested using 5 ml of sterile water per plate and the cell suspensions of all 5 plates were combined in a 50 ml centrifugation tube. 25 ml ethyl acetate was added to the suspension and the tube was vortexed for 3 minutes. The mixture was then centrifuged for 10 minutes at 1000 x *g* to facilitate separation of the aqueous and organic phase. The organic phase was recovered using a glass pipette and transferred to a glass vessel before the ethyl acetate was evaporated off under constant nitrogen flow. The result was resolved in ethanol and sterile filtered through a 0.22 µm filter. The filtered solution was then dried under constant nitrogen flow and weight before it was resuspended to 5 µg ml^{-1} in ethyl acetate.

3.3.7 Mass-spectrometric analysis

Mass spectrometric analysis of the biosurfactants was performed using a QTRAP 4500 (Applied Biosystems, AB Sciex) triple-quadrupole mass spectrometer, operated in negative electrospray ionization (ESI) - Q1 Scan Modus. The surfactant solution with a concentration

of 5 µg ml^{-1} was injected via a syringe pump set to a flow rate of 10 µl min-1 directly into the MS. The analytes were detected in negative mode within a mass over charge range of 1000 - 1200 m/z.

3.3.8 Plant growth and in planta experiments

Arabidopsis thaliana was grown axenically as described previously (Miebach et al., 2020). Briefly, Arabidopsis seeds were sterilised in a 1.5 ml Eppendorf tube by adding 1 mL 70 % ethanol and 0.1 % Triton X-100. The seeds were vortexed and then incubated for one minute. The supernatant was removed by pipetting, followed by the addition of 1 ml 10 % bleach and 10 µl of 0.1 % Triton X-100 for 12 minutes. After removing the bleach, the seeds were rinsed thrice with 1 ml of sterile distilled water and were stratified for 48 hours at 4 °C. Stratified seeds were pipetted onto Murashige and Skoog-agar (MS-agar, 2.2 g of Murashige and Skoog medium including vitamins (Duchefa) and 10 g plant agar (Duchefa) per litre of milliQ water, pH 5.8) filled 200 µL pipette tips that were shortened by 1 cm to allow the plant's roots to easily pass the tip. The tips were placed pointy end first into a MS-agar plate. The seeds were germinated for seven days at short day conditions (11 hours day/ 13 hours night). After the germination period, the seedling-filled tips were transferred to autoclaved Magenta™ GA-7 (bioWORLD) plant culture boxes filled with finely ground 90 g zeolite clay (Purrfit Clay Litter, Vitapet) and 60 ml MS medium. Four seedlings were transferred into each Magenta box and the plants were grown for an additional three weeks at short day conditions (11 hours day/ 13 hours night, chamber set to 85% relative humidity). To prepare bacterial inoculum, bacteria were cultured on LB broth overnight. Bacteria were then harvested by 10 min centrifugation at 2600 *g* and washed with 1 × phosphate buffer saline (PBS, 0.2 g L^{-1} NaCl, 1.44 g L^{-1} Na_2HPO_4 and 0.24 g L^{-1} KH_2PO_4). Bacteria were resuspended to an OD_{600nm} 0.5 and then serial diluted to OD_{600nm} 0.00005. For competition experiments wild type and surfactant knockout strains were mixed at a ratio of 1:1. 100 µL of the mix or the monocultures were inoculated onto three week-old Arabidopsis using an T-180 airbrush (KKmoon).

Bacteria were recovered by harvesting the leaf material of individual plants, placing them in a 1.5 ml Eppendorf vial. The plants were weighed and 1 mL 1 × PBS were added. The vial was vortexed for 2 minutes and then sonicated for 5 minutes in a sonication bath (Elmasonic) before they were vortexed for another 2 minutes. The supernatant was serial diluted and CFU of wild type and surfactant mutants were determined by growing the strains on LB agar containing appropriate antibiotics to select for either the spontaneous streptomycin resistant wild type or the kanamycin resistant mutants.

3.3.9 Hydrocarbon utilisation assay

To measure the ability of wild type and surfactant knockout mutants to grow on diesel or dodecane as the sole source of carbon, Bushnell-Haas broth (0.2 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2$, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} NH_4NO_3 and 0.05 g L^{-1} $FeCl_3$, pH 7.2), was supplemented with 1% diesel (commercial diesel, locally sourced) or 1 % dodecane (for synthesis, Merck) (Oso et al., 2019). Bushnell-Haas broth without additional carbon source was used as a negative control. In control experiments, to complement surfactant knockout mutants, between 0.23-0.265 mg mL^{-1} of isolated WT surfactants or 0.1 mg mL^{-1} Tween-20 were supplemented. Bacteria were grown overnight in LB, diluted 100 × using Bushnell-Haas broth without carbon source. The diluted bacterial suspensions were inoculated into 50 mL broth cultures in 250 mL Erlenmeyer flasks. Cultures were incubated at 30°C and 200 revolutions per minute for up to 17 days. Cell density was regularly measured by determining the optical density at 600 nm using a spectrophotometer (Biochrom WPA CO8000, Biowave). All experiments were performed in three biological replicates.

3.3.10 Statistical analysis

Statistical analysis was performed using Prism 9 (Graphpad). To analyse growth data in liquid culture, two-way ANOVAs with Tukey's multiple comparisons test were performed. To analyse wild type and corresponding knockout mutant growth *in planta*, two-way ANOVA with Šídák's multiple comparisons test was performed.

3.4 Results

3.4.1 Phylogenetic placement of *Pseudomonas* sp. FF1, FF2, FF3 and FF4

Analysis of the 16S rRNA genes of all four isolates revealed that they are all members of the genus Pseudomonas and members of the *Pseudomonas fluorescens* lineage and subgroup (Peix et al., 2018). *P*FF1 clusters closely with *Pseudomonas orientalis*, *P*FF2 clusters closely with *Pseudomonas extremaustralis*, while *P*FF3 and *P*FF4 cluster closely with *Pseudomonas paralactis* (Figure 3-1). *P*FF1 and *P*FF2 are closer related to each other than to *P*FF3 and *P*FF4. *P*FF3 and *P*FF4 are closely related.

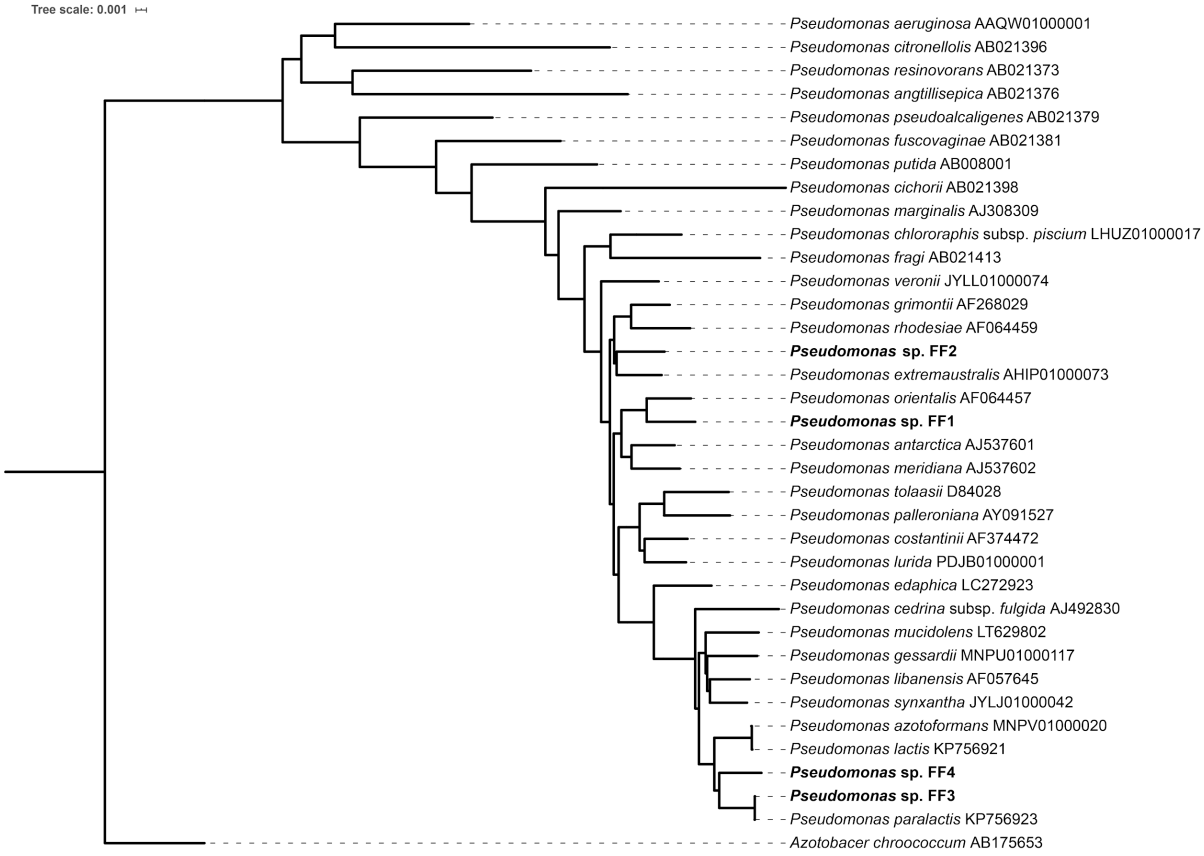

Figure 3-1 Phylogenetic placement of the four isolated Pseudomonads.
The newly sequenced isolates are highlighted in bold. NCBI accession numbers of the respective sequences are noted behind the species names. Azotobacter chroococcum was used as an outgroup.

3.4.2 Surfactant production of tested Pseudomonads

All four wild type Pseudomonads were tested for their production of surfactants on agar plates using the atomised oil assay (Burch et al., 2010; Oso et al., 2019). All four strains produced clear halos where the reflection of the oil to light changed indicating production of surfactants (Figure 3-2A-D). Similarly, the positive control Tween-20 showed a halo (Figure

3-2E), while the negative control, *Escherichia coli* DH5α, was lacking a halo (Figure 3-2F). The drop collapse assay was used as a secondary test for surfactant production. All tested wild type culture supernatants collapsed into the engine oil (Figure 3-2G-J). The collapse is due to a change in surface tension of the supernatant.

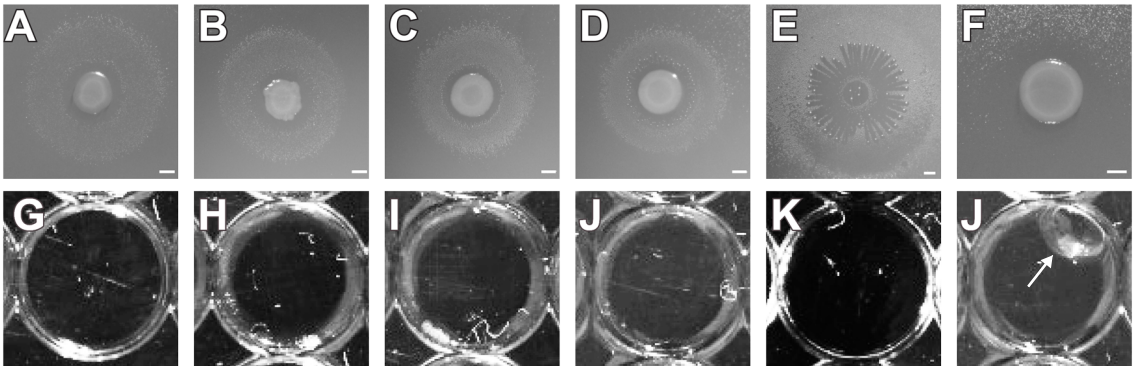

Figure 3-2 A-F) Atomised oil assays to demonstrate the production of surfactants. A-D) wild type colonies of PFF1, PFF2, PFF3, and PFF4, respectively, exhibiting a halo indicative for surfactant production. E) Tween-20 F) E. coli DH5α *G-L) Drop collapse assays to demonstrate the production of surfactants. Culture supernatants of wild type PFF1, PFF2, PFF3, and PFF4, respectively, collapsed into oil indicative for surfactant production. K) collapsed drop containing Tween-20. J) Non-collapsed drop of E. coli culture supernatant. The non-collapsed drop is highlighted by an arrow.*

3.4.3 Mass spectrometric analysis of surfactants

The analysis of surfactants harvested from the Pseudomonads using LC-MS with ESI in negative mode revealed that *P*FF1 and *P*FF2 produced the same compounds with a characteristic main peak at m/z=1124.59 (Figure 3-3A and B), which can be attributed to the deprotonated molecular ion $[M-H]^-$. The analogous pattern for the protonated molecular ion $[M+H]^+$ has been previously described for the cyclic lipopeptide viscosin when using ESI in positive mode for detection (De Bruijn et al., 2008; Laycock et al., 1991). Similarly, *P*FF3 and *P*FF4 share the same characteristic main peak at m/z=1138.60 (Figure 3-3C and D), the analogous pattern has previously been described for the cyclic lipopeptide massetolide A (De Bruijn et al., 2008).

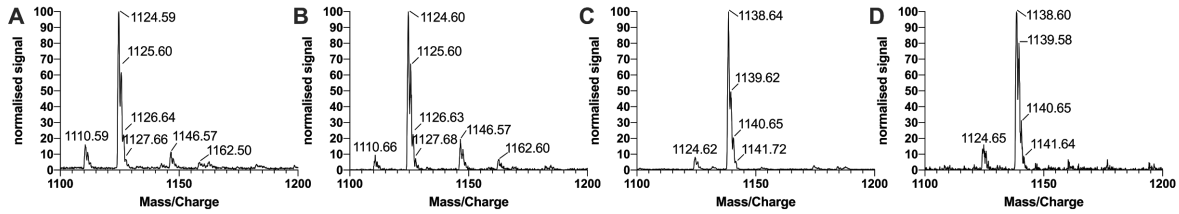

Figure 3-3 (A-D) MS/MS spectra of extracted surfactants of PFF1, PFF2, PFF3, and PFF4 respectively. PFF1 and PFF2 both produce viscosin-like surfactants, PFF3 and PFF4 both produce massetolide A-like surfactants. Spectra were normalised against the maximal intensity.

3.4.4 Random Tn*5* mutagenesis and mutant characterisation

The surfactants producing wild types were subjected to random insertion mutagenesis using the EZ-Tn5 transposon system. The screen resulted in a transposon mutant library with several hundred transposon mutants for each of the four isolates. We obtained 3 in 168, 4 in 1100, 26 in 1725, and 1 in ~200 surfactant negative mutants for *P*FF1, *P*FF2, *P*FF3 and *P*FF4, respectively. *Pseudomonas* genomes are between 6 Mbp and 6.5 Mbp in size, based on the size of previously published sizes of viscosin and massetolide A gene clusters (each ~ 30 Kbp) (De Bruijn et al., 2008; De Bruijn et al., 2007), we were expecting approximately 1 surfactant negative mutant every ~200-220 clones not considering essential genes. Each of the mutant libraries was screened with the atomised oil assay for lack of surfactant production mutants. For each strain, we selected one of the obtained surfactant mutants for further characterisation studies (Figure 3-4A-D). The drop collapse assay was conducted and confirmed the results of the atomised oil assay (Figure 3-4E-H). The insertion site of each mutant was determined by digesting the genomic DNA of the mutants and cloning it into pUC19 before selecting for kanamycin resistance encoded in the transposon (Supplemental Table 1).

The investigated *P*FF1 mutant carried an insertion in a gene with 97% similarity to a non-ribosomal peptide synthetase in *P. orientalis* F9 (Genbank: BOP93_14875) (Zengerer et al., 2018) which has an 80% peptide similarity to the *viscB* gene of *P. fluorescens* SBW25 (UniProtKB ID: C3K9G2) (De Bruijn et al., 2007; Silby et al., 2009). The investigated *P*FF2 mutant carried an insertion in a gene with an 86% similarity to the *viscB* gene (Genbank: CAY48788.1) of *P. fluorescens* SBW25, respectively. Therefore, they are designated

*P*FF1::ezTn5-viscB and *P*FF2::ezTn5-viscB, respectively. The *viscB* gene encodes for a non-ribosomal peptide synthetase that, in conjunction with *viscA* and *viscC*, produces the cyclic lipopeptide biosurfactant viscosin (De Bruijn et al., 2007). The *P*FF3 Tn*5* transposon mutant carried an insertion in a gene with 99% similarity to the *massB* gene in *Pseudomonas fluorescens* SS101 (Genbank: ABH06368.2). The *P*FF4 Tn*5* transposon mutant carried an insertion in a gene with 95% similarity to the *massB* gene in *Pseudomonas fluorescens* SS101 (De Bruijn et al., 2008). The *massB* gene is part of the massetolide A synthesis gene cluster. Therefore, the mutants were designated *P*FF3::ezTn5-massB and *P*FF4::ezTn5-massB.

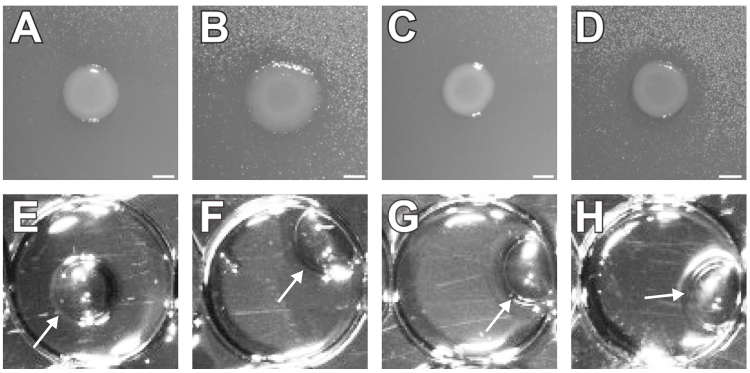

Figure 3-4 A-D) Atomised oil assay to demonstrate the production of surfactants. Tn5-transposon insertion mutant colonies PFF1::ezTn5-visB, PFF2::ezTn5-visB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB, respectively, lacking a halo indicative for surfactant production. E-H) drop collapse assays to demonstrate the production of surfactants. Culture supernatants of Tn5-transposon insertion mutant PFF1::ezTn5-visB, PFF2::ezTn5-visB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB, respectively, showing a beaded bubble swimming on top of oil, indicative for the lack of surfactants. The non-collapsed droplets are highlighted by arrows.

After performing the surfactant extraction protocol from mutant inoculated agar plates, no surfactants could be detected (Figure 3-5 A-D).

The effect of the transposon insertions and the lack of surfactant production was tested in shaking liquid cultures in two different conditions, either KB complex medium (Supplemental Figure 1A), or M9 minimal medium supplemented with glucose as the sole source of carbon (Supplemental Figure 1B). None of the tested insertion mutants exhibited significantly changed doubling times under the two tested conditions.

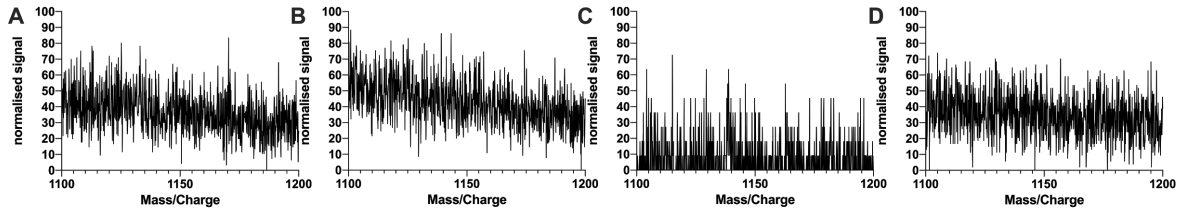

Figure 3-5 A-D) Knockout mutants show no sign of surfactant production. MS/MS spectra of extracts of PFF1::ezTn5-viscB, PFF2::ezTn5-viscB, PFF3::ezTn5-massB, and PFF4::ezTn5-massB, respectively. None of the random knockout mutants produced detectable surfactant peaks at the respective wild type m/z values. Spectra were normalised against the maximal intensity.

3.4.5 Growth on diesel oil or dodecane as sole carbon source

To investigate if the lack of surfactant production could impact the ability of the Pseudomonad strains to degrade alkanes, the different wild types and transposon mutants were grown on Bushnell-Haas broth with diesel as the sole carbon source. This experiment revealed that all surfactant mutants, even though they were still able to grow on diesel, had a reduced growth rate, and a reduced final optical density after up to 21 days of growth (Figure 3-6). No growth could be observed on Bushnell-Haas broth without carbon source for either the wild type or the surfactant mutants. In general, the growth on diesel oil was slower compared to growth on complex medium or minimal medium supplemented with glucose as sole carbon source and better described by a linear function than an exponential growth function. By supplementing knockout mutants with biosurfactants harvested from respective wild type strains or the synthetic surfactant Tween-20, growth on diesel could be complemented in parts, or completely, compared to the wild type. The knockout mutants were not able to grow on surfactants alone to a degree that explains the increased growth on diesel (Supplemental figure 2).

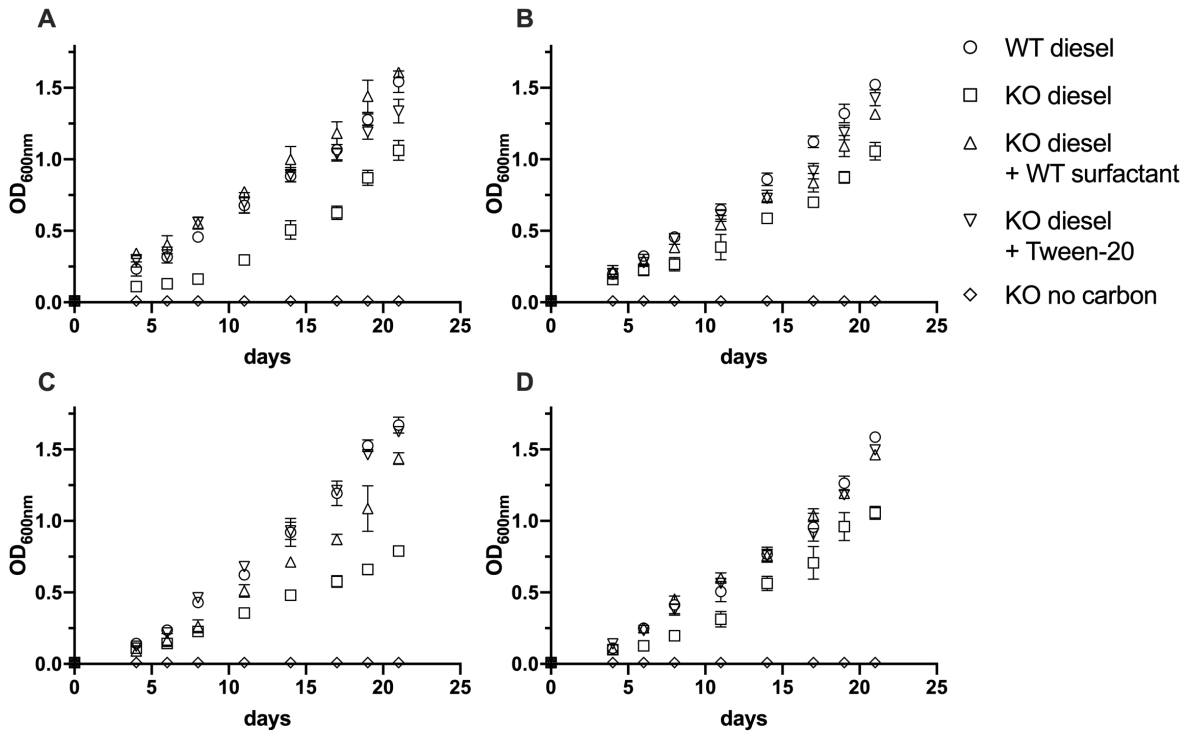

Figure 3-6 Utilisation of diesel by biosurfactant knockout mutants and wild types.
A) PFF1, B) PFF2, C) PFF3, D) PFF4. Each wild type and knockout mutant was grown in Bushnell-Haas broth supplemented with diesel as the sole source of carbon (circle and square, respectively). Knockout mutants were complemented with either wild type surfactant (triangle), Tween-20 (inverted triangle) or were incubated with no additional carbon source (diamond). Error bars depict the standard deviation of the mean. Experiments were performed in triplicates.

While growing on dodecane, surfactant production had a less dramatic effect compared to growth on diesel (Supplemental figure 3 and Supplemental table 3). The overall growth was lower and the maximal OD600nm of all tested strains was more than a magnitude lower compared to the growth on diesel and the maximal OD600nm was reached after 9 - 13 days. After 21 days, the optical densities were already markedly reduced and the cultures were in their death phase. For all wild type and mutant combinations, we were able to detect periods where the wild type achieved was significantly higher optical densities compared to the knockout mutants.

3.4.6 Fitness in planta

To investigate changes in the ability of the transposon mutants to colonise leaf surfaces, the mutants were co-inoculated with the respective wild types by airbrushing. Whole above-ground plant material was sampled daily for six days and colony forming units of wild type

and transposon mutants were determined (Figure 3-7). The initial bacterial densities were similar between wild type and knockout mutants. Wild types (*P*FF1, *P*PF2, *P*PF3 and *P*PF4) and corresponding mutants (*P*FF1::ezTn5-viscB, *P*FF2::ezTn5-viscB, *P*FF3::ezTn5-massB and *P*FF4::ezTn5-massB) colonised Arabidopsis at similar rates. *P*PF1, *P*PF2 and their mutants reached approximately 10^7 CFU per gram of plant weight, whereas *P*FF3, *P*PF4 and their mutants reached approximately 10^6 CFU per gram of plant weight. Thus, no differences between the plant colonisation of wild type and mutants were found. Furthermore, growth in planta of all strains was tested individually, no significant differences in plant colonisation could be determined (Supplemental figure 3).

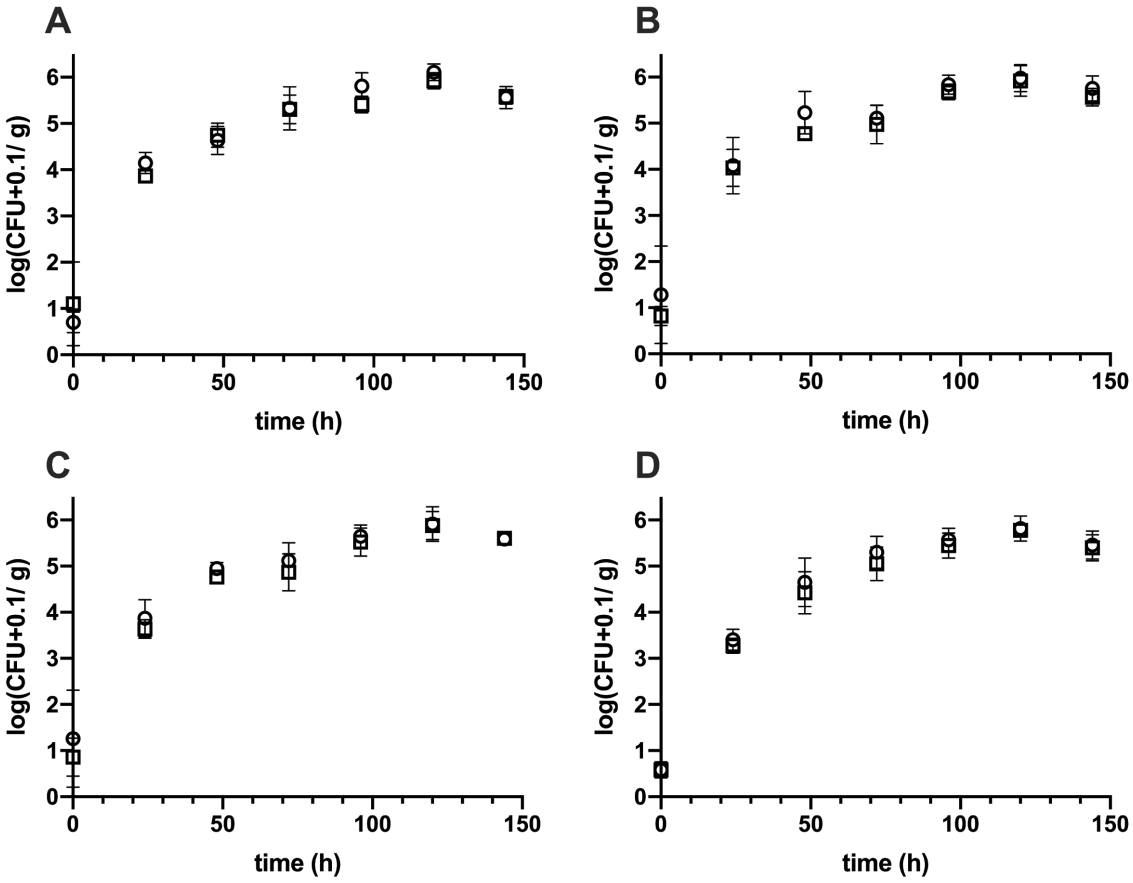

Figure 3-7 In planta competition of wild types (open circles) and mutants (open squares). A) PFF1 vs. PFF1::ezTn5-visB, B) PFF2 vs. PFF2::ezTn5-visB, C) PFF3 vs. PFF3::ezTn5-massB, D) PFF4 vs. PFF2::ezTn5-massB. Symbols represent the mean CFU on five plants per measurement. Error bars depict the standard deviation of the mean. Experiments were performed in quintuplets.

3.5 Discussion

All four Pseudomonads isolated from either spinach or romaine lettuce leaf material (Burch et al., 2016) belong to the fluorescent Pseudomonads (Gomila et al., 2015). *P*FF1 and *P*FF2 are phylogenetically more closely related to each other than to *P*FF3 and *P*FF4. *P*FF3 and *P*FF4 are very closely related. All four strains are produced surfactants on agar plates and in liquid culture as shown by the atomised oil assay and the drop collapse assay. As the ability to produce surfactants is widely distributed in the genus *Pseudomonas*, this result was not surprising (Geudens & Martins, 2018; Nybroe & Sørensen, 2004). The relatedness of the strains is also reflected in the surfactants that each of the strains is producing: *P*FF1 and *P*FF2 produce the viscosin-like surfactants, while *P*FF3 and *P*FF4 are produced massetolide A-like surfactants. The production of viscosin and massetolide A by Pseudomonads has been demonstrated previously (De Bruijn et al., 2008). Both viscosin and massetolide A are the product of nonribosomal peptide synthetase genes. Viscosin production depends on a gene cluster encompassing the three genes *viscA*, *viscB*, *and viscC* and which spans approximately 32 kb (De Bruijn et al., 2007). Massetolide A production also depends on a gene cluster which encompasses the three genes *massA*, *massB* and *massC* and spans approximately 30 kb (De Bruijn et al., 2008).

To further investigate the ecological function of the surfactants in the leaf colonising Pseudomonads, random Tn*5* transposon insertion mutants were produced and further characterised. The screen yielded complete loss of surfactant production mutants for every strain, indicating that each strain only encodes for one surfactant that is active during the selection conditions. The insertion sites were mapped to genes that matched previously characterised non-ribosomal peptide synthase clusters responsible for surfactant production, and which matched the surfactants that were identified using mass-spectrometry. *P*FF1 and *P*FF2 knockout mutants were mapped to *viscB* gene homologues, and *P*FF3 and *P*FF4 knockout to *massB* gene homologues (De Bruijn et al., 2007; De Bruijn et al., 2008).

The assumption that only one surfactant is produced by each strain was corroborated by a sequence of experiments during which the surfactant mutants consistently failed to produce

signs of surfactant production independent of their growth conditions. The surfactant mutants failed to produce halos in the atomised oil assay, and the culture supernatant did not collapse into motor oil in the drop collapse assay. Mass spectrometric analysis of the knockout mutants showed that the production of surfactants was completely abolished and no detectable peak pattern was found after the surfactant extraction procedure (Figure 3-5).

Despite the loss of surfactant production and the additional burden of expressing the kanamycin resistance gene from the Tn*5* transposon, the insertions had no detectable fitness effects in either complex KB medium or minimal M9 medium supplemented with glucose. In shaking liquid cultures, surfactants did not provide critical functions for growth (Supplemental figure 1). We hypothesise that surfactants may enable bacteria to utilise parts of the plant cuticle as a source for carbon. Even though it was not possible to show that Pseudomonads and their respective mutants had differential abilities to utilise hydrocarbon components from isolated cuticles (data not shown), a clear difference in the ability of wild type and mutant to utilise diesel for growth was demonstrated (Figure 3-6). Even though growth was not completely abolished, it was significantly reduced (Supplemental table 2). This could also explain why growth on isolated cuticles did not yield conclusive results and differences between wild type and knockout mutant. Due to the size of the non-ribosomal peptide synthetase genes, it was not possible to construct rescue mutants. However, we attempted to complement the reduced ability of the knockout mutants to degrade diesel oil by adding harvested wild type surfactant or Tween-20 to growing cultures. Indeed, both surfactants were able to complement the growth phenotype either in parts or completely (Figure 3-6 and Supplemental table 2), evidencing that the lack of surfactants was the causal reason for reduced growth. Despite the chain length differences between the diesel (Wante & Leung, 2018) and the alkane monomers in waxes of leaf cuticles (Zeisler-Diehl et al., 2018), the chemistry of both aliphatic mixtures contain similar monomers. It is thus not unthinkable that, under nutrient limiting conditions, the *Pseudomonas* strains tested here are able to utilise aliphatic components of leaf cuticles in a surfactant-dependent manner.

However, we failed to provide a final proof of this relationship (details of the experiments conducted for this study can be found in appendix 3).

To investigate the role of the surfactants during plant colonisation we inoculated axenically grown Arabidopsis with mixtures of wild type and knockout mutants or with individual strains. During co-inoculation with their respective wild types onto axenic Arabidopsis, no fitness disadvantages for the knockout mutants were detected. This might be a consequence of the surfactant acting as a public good that increases the fitness of wild type and co-inoculated mutants alike (Lyons & Kolter, 2017). However, single strain inoculations also did not result in a diminished ability of the knockout mutants to colonise Arabidopsis. This is in contrast to previous experiments that demonstrated that surfactants do indeed have a positive effect on plant colonisation (Burch et al., 2014). It is noteworthy that the experimental setup used in our study was markedly different including a different plant host as well as incubation conditions under constant relative humidities. While previously it was shown that fluctuating humidities are a prerequisite to result in a fitness advantage. Therefore, it might still be possible the surfactants in the here-tested strains will impact plant colonisation for example under fluctuating relative humidities, by increasing mobility of the strains on the phylloplane (Burch et al., 2012; Raaijmakers et al., 2010), or increasing permeability of leaf cuticles (Schreiber et al., 2005). Furthermore, the antimicrobial activity of many surfactants may provide fitness advantages in microbial communities (Raaijmakers et al., 2010).

3.6 Conclusion

The experiments reported here demonstrated that the biosurfactants produced by four different leaf colonising Pseudomonads impacted on their ability to degrade aliphatic compounds. However, the ability to produce biosurfactants had no measurable impact on the ability of the strains to colonise axenic Arabidopsis leaves in competition or after individual strain inoculations. We gathered additional evidence that the bacteria may utilise aliphatic compounds originating from leaf cuticles but failed to conclusively demonstrate a

relationship between surfactant production and leaf colonisation ability. Future studies will have to be performed to address this hypothesis.

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4 The presence of surfactant genes does not improve diesel degradation by epiphytic Pseudomonads in soil.

4.1 Abstract

Pseudomonas strains isolated from plant leaf surfaces and their surfactant mutants were investigated for their ability to degrade diesel in soil. Both the wild types and their respective mutants were able to grow better in diesel-supplemented soil microcosms compared to diesel free control soil. There was no detectable difference between wild type and surfactant mutant populations growing on diesel-supplemented soil. To further investigate the amount of diesel degraded by the wild type and mutant strains, gas chromatography coupled with flame ionisation detection was performed, revealing that wild types and mutants were able to degrade diesel to a similar degree. This result was unexpected since previous results demonstrated that surfactant mutants did not grow as well as wild types in minimal media supplemented with diesel.

4.2 Introduction

Hydrocarbons are known to be recalcitrant to degradation and may therefore persist in the environment. This is due to their hydrophobicity and low bioavailability (Atlas & Atlas, 1995; Trindade et al., 2005). Several bacterial genera inhabiting leaf surfaces have been shown to utilise hydrocarbons. Examples of these bacterial genera are *Aeromicrobium*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Erwinia*, *Flavobacterium*, *Lactobacillus*, *Methylobacterium*, *Pseudomonas, Rhodococcus*, *Sphingomonas*, and *Xanthomonas* (Atlas et al., 1992; Okoh et al., 2001; Oso et al., 2019; Sarkar et al., 2017; Scheublin & Leveau, 2013; Xu et al., 2017). Amongst these, the Pseudomonads are one of the best studied hydrocarbon utilising bacteria (Oso et al., 2020; Oso et al., 2019; Pacwa-Płociniczak et al., 2014; Palleroni et al., 2010; Wu et al., 2018; Zhang et al., 2011). Some bacteria produce surfactants which may facilitate access to these hydrocarbons as sources of carbon and energy (Leuchtle et al., 2015). In Chapter 3 for example, the surfactant producing, leaf-isolated Pseudomonads PFF1, PFF2, PFF3 and PFF4 were shown to be hydrocarbon utilisers. This was evident when the ability of the individual wild type strains and the surfactant mutant strains was tested in minimal medium supplemented with diesel as sole source of carbon (details in Chapter 3). Results from the study showed a differential ability to utilise diesel with the growth of surfactant mutant strains markedly reduced in comparison to the wild type strains. Surfactants have been shown to enhance the degradation of hydrocarbons by increasing the surface area between immiscible liquids and increasing the pseudo-solubility by partitioning oils into micelles. This can increase the bioavailability of hydrocarbons for microorganisms (Pacwa-Płociniczak et al., 2011).

Leaf surfaces are also a source of surfactant producing bacteria (Burch et al., 2010; Oso et al., 2019). Leaf colonising bacteria are extremely hardy and thrive despite being exposed to many detrimental factors such as fluctuating temperature, UV radiation and relative humidity (Lindow & Brandl, 2003; Schlechter et al., 2019; Vorholt, 2012). Additionally, many of these bacteria e.g., *Pseudomonas* spp. are able to utilise aliphatic compounds (Burch et al., 2010;

Oso et al., 2019). The combination of these abilities i.e. surfactant production, survival under harsh conditions, and the ability to degrade hydrocarbons could make epiphytic bacteria good candidates for bioremediation of oil contaminated environments such as soil.

To investigate hydrocarbon remediation in soil, several methods can be used (Anonymous 1999). These methods have in common that hydrocarbons are extracted from environmental samples using solvents. Hydrocarbons are then determined by gravimetry, infrared spectroscopy or gas chromatography (GC) with different detection modes, e.g. flame ionisation detection (FID) or mass spectrometry (MS) (Wang et al., 1999). GC methods compare a standard chromatographic response i.e. the overall peak area to the chromatogram response of the sample. GC coupled to FID (GC-FID) is the most prevalent method to measure hydrocarbons in environmental samples. The reasons for this are low detection limits, low interference by naturally occurring substances and the ability to provide reliable concentration measurements of individual hydrocarbons. GC-FID can also measure the total petroleum hydrocarbons (Frysinger et al., 2003; Krupcík et al., 2004; Risdon et al., 2008; Wang & Fingas, 2003).

In Chapter 3, phyllosphere colonising Pseudomonads were shown to utilise hydrocarbons as sources of carbon and energy and their ability to produce surfactants impacted the efficacy of hydrocarbon utilisation. In this chapter, the ability of phyllosphere colonising Pseudomonads to degrade hydrocarbon in soil was assessed, as was the impact of surfactant production on the degree of degradation. Bioremediation experiments were performed by inoculating bacteria into diesel-contaminated soil. Bacterial growth was monitored and diesel degradation was determined using GC-FID. The results obtained from this study may contribute to the selection of organisms for environmental bioremediation.

4.3 Materials and methods

4.3.1 Strains and culture conditions

All Pseudomonads and their surfactant mutants (*Pseudomonas* sp. (PFF1), PFF1::ezTn5-viscA (PFF1 mut), *Pseudomonas* sp. (PFF2), PFF2::ezTn5-viscA (PFF2 mut), *Pseudomonas* sp. (PFF3), PFF3::ezTn5-massA (PFF3 mut), *Pseudomonas* sp. (PFF4), PFF4::ezTn5-massA (PFF4 mut) used in this study were routinely cultivated on Nutrient agar (NA, Fort Richards laboratories) at 30℃. R2A agar (Hi-media) was used to check for sterility of soil. To prepare bacterial inoculum for hydrocarbon degradation experiments, bacteria were cultivated in Bushnell Haas broth (BHB, 0.2 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2$, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} NH_4NO_3 and 0.05 g L^{-1} $FeCl_3$, pH 7.2) supplemented with glucose.

4.3.2 Preparation of soil microcosms

Soil was collected from the green house of the University of Canterbury and sieved through a 1 mm screen before use. Soil was sterilised by autoclaving at 121℃ and kept at room temperature for 7 days to allow for spore forming bacteria to germinate. The autoclaving procedure was repeated three times. Sterility of the soil was confirmed by plating 100 µl aliquots of soil slurry on Reasoner's 2A agar (HiMedia). The soil microcosms were prepared using glass culture tubes with Teflon lined screw caps (Kimax) each containing 5 g of sterile pre-dried soil. 1.5 ml of sterile distilled water was added to the soil before inoculating with bacteria to improve moisture content before supplementing with 50 µl of locally-sourced diesel obtained from (Wante & Leung, 2018).

Before bacteria were inoculated into soil supplemented with diesel, a single bacterial colony was picked with a sterile toothpick and inoculated into 50 ml of BHB supplemented with 1% w/v glucose. The cultures were incubated overnight at 30℃ and 200 revolutions per minute (rpm). Bacterial cells were harvested by centrifugation at 2600 × *g* for 10 minutes and were resuspended in 50 ml 1 × PBS. This procedure was repeated twice. Soil microcosms were

inoculated with 50 µl washed culture containing 1 x 10^8 cfu/ml$^-$. The following treatments were prepared in triplicates and were incubated at 30℃ for 35 days and bacterial colony forming units (CFU) were determined every 5 days: 1) Diesel-supplemented soil inoculated with wild type strains, 2) Diesel-supplemented soil inoculated with knockout strains, 3) Soil inoculated with wild type strains, 4) Soil inoculated with knockout mutant strain, and 5) Control tubes with diesel-supplemented soil without inoculation and non-contaminated soil without inoculation.

4.3.3 Recovery of bacteria from soil

To determine CFU in soil, 1 g of soil material was collected from the from the 5 g soil in the microcosm into a sterile falcon tube using a sterile spatula, 9 ml of sterile distilled water were added and the mixture vortexed. 100 µl were removed for serial dilution and CFUs were determined after growth on NA plates. The leftover 4 g of soil were used to recover hydrocarbons as described below.

4.3.4 Recovery of hydrocarbons from soil

To extract diesel from contaminated soil, 15 ml of pentane (BDH laboratories, F chemicals, or VWR chemicals) and 100 µl of 1 mg ml^{-1} 5-α androstane (Sigma), which served as internal standard (IS), were added to the remaining 4 g of soil from the soil microcosm in a 40 ml Kimax glass tube (Kimble). The mixture was vortexed vigorously and allowed to stand to allow for debris to settle. Using a Whatman No. 1 filter paper and glass funnel, the mixture was filtered into a 20 ml screw-thread storage vial. The pentane was evaporated under a stream of nitrogen in a fume hood. 15 ml of n-pentane were added into the 20 ml screw-thread storage vial (Interlab, Wellington, New Zealand) containing the residual diesel. 1 ml of the solvent mixture was then transferred to a 1.5 ml amber glass vial with screw caps fitted with PTFE silicon-septa (Interlab, Wellington, New Zealand). The samples were kept at 4℃ until they were analysed.

4.3.5 GC-FID analysis

The residual oil was analysed using a GC-2010 GC-FID (Shimadzu) equipped with a BPX5 (Trajan SGE GC column, 0.32 mm × 0.25 µm × 15 m). The carrier gas was nitrogen. The column temperature was held at 50℃ for 5 minutes. Afterwards, the temperature was increased by 10℃/min and held at 300℃ for 5 minutes. The injector temperature was set to 300℃. Results were recorded using the GC2010 GC Solution software (Shimadzu).

4.3.6 Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare the log transformed CFU/g across the wild type and surfactant mutant strains. Analysis was carried out in Prism 8.2.0 (GraphPad). To analyse the data from the GC-FID, an ordinary one-way ANOVA was used to compare diesel degradation by wild type and surfactant mutant Pseudomonad strains.

4.4 Results

4.4.1 Physicochemical analysis of soil

The soil had a pH of 5.3. The total Olsen phosphorus, magnesium, sodium, total carbon, total nitrogen and organic matter were low while the calcium content was high. The potentially available nitrogen was in the medium range (100 – 200 Kg/ha). Additional soil characteristics can be found in Supplemental Table 2.

4.4.2 Growth of epiphytic Pseudomonads in diesel-contaminated soil

To determine the effect of surfactants on the degradation of diesel, the wild type and surfactant mutant strains were grown on diesel-contaminated soil with CFU determined for 35 days (Figure 4-1). The bacterial populations increased for both wild type and surfactant mutant strains from 10^1 to 10^7 CFU/g with PFF3 wild type having the highest population of 4.2×10^7 CFU/g at 30 days. Bacterial population development, as measured by CFU $g^{-1}$of the tested Pseudomonads was significantly higher in the contaminated soil microcosm compared to the uncontaminated soil microcosm. Bacterial growth in diesel-supplemented

microcosms was significantly higher compared to non-diesel supplemented controls (two way ANOVA with multiple comparison test; Supplemental Table 3). By contrast, population development of wild type and surfactants mutant strains was not significantly different. As expected, there was no measurable growth observed in uninoculated control tubes. From the 50 µl of diesel added to each microcosm, 20-30 µl were recovered. No oil was recovered from non-contaminated microcosms i.e., inoculated control microcosms without diesel.

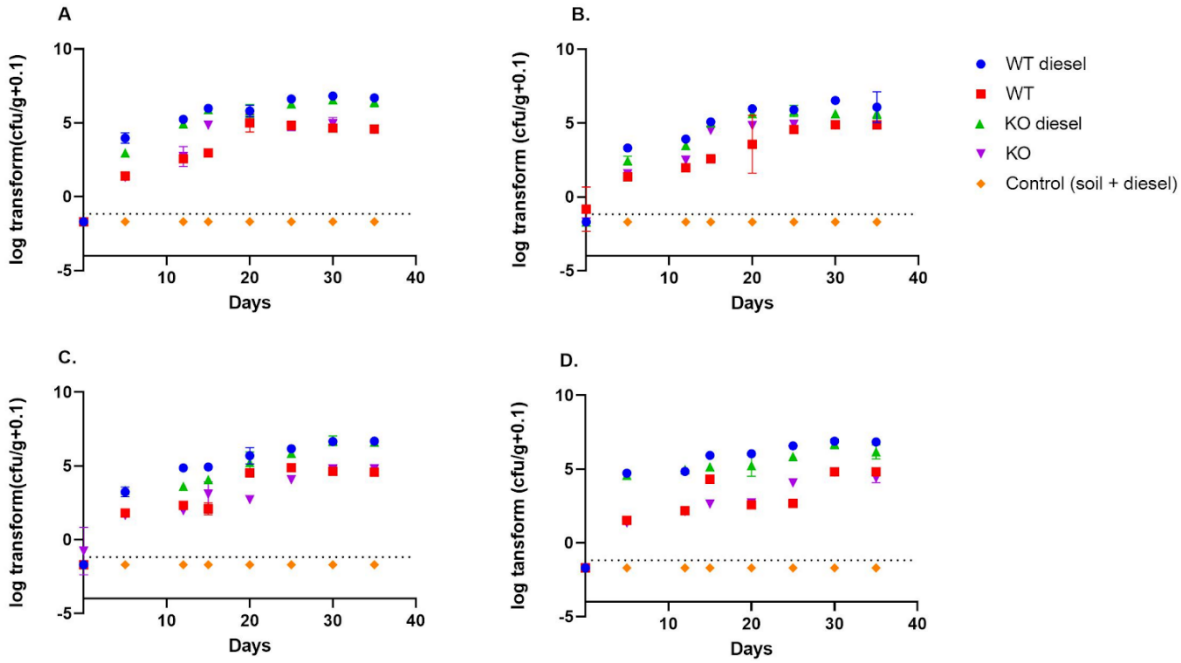

Figure 4-1 Bacterial CFU in soil microcosms.
A) PFF1 vs. PFF1::ezTn5-visB, B) PFF2 vs. PFF2::ezTn5-visB, C) PFF3 vs. PFF3::ezTn5-massB, D) PFF4 vs. PFF2::ezTn5-massB. Wild types grown with diesel as sole carbon source are depicted by circles, wild type without carbon source are depicted with squares, knockout mutants with diesel as sole carbon source by triangles, wild type without carbon source by inverted triangle and control by diamonds. No significant differences were found between the wild types and surfactant mutant strains (two-way ANOVA, Tukey's multiple comparisons test) as the wild type and surfactant mutant stains degraded diesel to a similar degree. Error bars depict the standard deviation of the mean.

4.4.3 Gas chromatographic analysis of residual diesel

After 35 days, a GC-FID analysis of the residual diesel in the soil was performed and bacterial treated samples were compared to mock treatments. There was no significant difference in the residual diesel between the wild type and respective surfactant mutant

strains (Figure 4-2). Furthermore, no significant difference between the total residual amounts of diesel between the different bacterial strains was detected (Figure 4-3). Hence, all the strains were able to degrade diesel in soil to a similar degree.

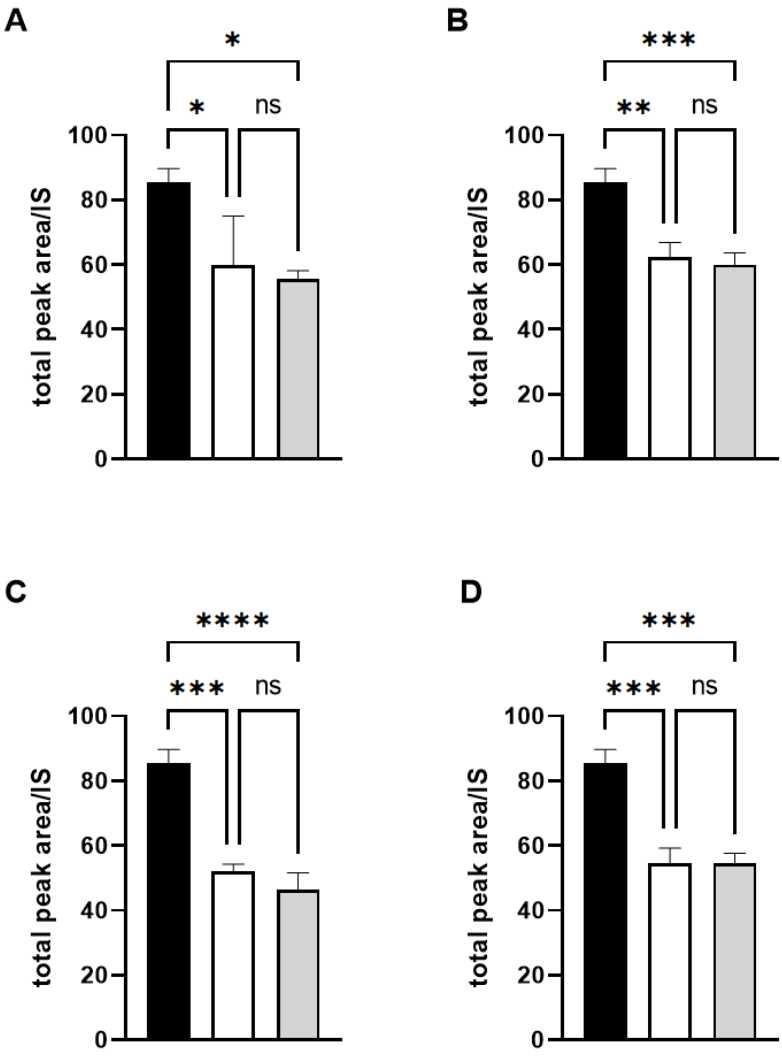

Figure 4-2 Bacterial degradation of diesel in soil microcosms after 35 days.
*Total peak area was normalised against the internal standard 5-α androstane. (A) PFF1 (B) PFF2 (C) PFF3 (D) PFF4. Mock treatments are depicted in black, wild type treatments in white and mutant treatments in light grey. *, represent different levels of significance (* = p < 0.05, ** =p< 0.01, *** = P< 0.001, **** P<0.0001). No significant difference was found between the wild type and surfactant mutant strains (ordinary one-way ANOVA, Tukey's multiple comparisons test P<0.05) as the wild type and surfactant mutant strains degraded diesel to a similar degree. There was however a significant difference between the individual strains growing on diesel contaminated soil and mock treatment. Error bars depict the standard deviation of the mean.*

The chromatograms show the ability of the bacterial strains to degrade diesel (Supplemental figure 5). Although the differences when comparing chromatograms of diesel-contaminated

samples inoculated with bacteria to mock inoculated samples were inconclusive, peaks at retention times between 10 to 10.2 minutes are lower and peaks at retention times between 10.2 to 10.5 minutes were lacking. Furthermore, peaks at retention times between 11.74 to 11.9 minutes were lacking in bacteria inoculated samples. Additional peaks were observed at retention time retention times 14.2 and between 24.25 - 25.50 minutes. The degradation of these peaks happened at almost the same retention time for all the wild type and surfactant mutant strains.

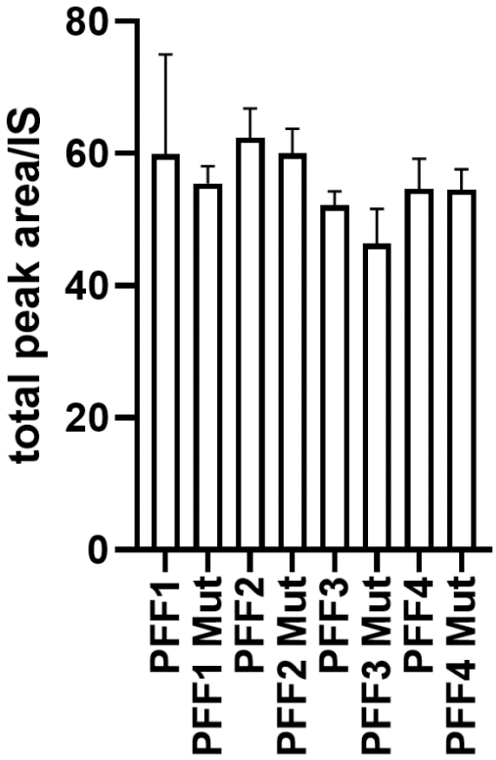

Figure 4-3 Comparison of diesel degradation by wild type and surfactant mutant Pseudomonas strains.
No significant difference was found between the wild type and surfactant mutant strains as the wild type and surfactant mutant strains degraded diesel to a similar degree (ordinary one-way ANOVA, Tukey's multiple comparisons test P<0.05). Error bars depict the standard deviation of the mean.

4.5 Discussion

In this part of the study, the role of surfactants in the degradation of hydrocarbons in heterogeneous soil environments was investigated. The results show that the tested epiphytic Pseudomonads were able to degrade diesel. This is in line with other studies that have shown that Pseudomonads are effective at degrading hydrocarbons in different environments (Marchut-Mikolajczyk et al., 2018; Patowary et al., 2017; Wu et al., 2018).

However, the data also showed that there were no significant differences between wild types and their respective surfactant mutants while growing on soil. This suggests that the presence of surfactant production genes in the soil microcosm did not increase diesel availability to the bacterial populations. This contrasts with previous studies that have shown that the exogenous addition of surfactants produced by different Pseudomonads during the remediation of hydrocarbons resulted in higher degradation efficiency (Beal & Betts, 2000; Grimberg et al., 1996; Pacwa-Płociniczak et al., 2014).

The results in soil are also in contrast to results presented in Chapter 3 that demonstrated a significant effect between the wild type and surfactant mutant strains in their ability to utilise diesel in liquid medium (Oso et al., 2020). Currently, it is unclear if the *Pseudomonas* wild types were actively expressing their surfactant genes in the soil microcosm and whether reduced or no expression explains the lack of growth advantage. The reasons for the similarity in the degradation ability between the wild type and surfactant mutant strains tested in soil might be due the physico-chemical conditions of the soil and other environmental factors.

Diesel contains different hydrocarbon compounds as shown in chapter 1. This potentially may have supported the growth of the mutant strains as the corresponding chromatograms do not provide evidence that wild types and mutants were utilising different subfractions of the diesel. Many hydrocarbon degrading bacteria such as Pseudomonads possess the *alkB* gene which is important for hydrocarbon degradation. The *alkB* gene encoded Alpha-

ketoglutarate-dependent dioxygenase enables the utilisation of n-alkanes ranging from C_{10}-C_{16} (van Beilen and Funhoff, 2007; Smith et al., 2002). Surfactant mutants could utilise C_{10}-C_{16} components of the diesel as the wild type did. The reason for this could be the presence of the a*lkB* gene which is responsible for the degradation of those compounds. This may be why there was no significant difference between the wild type and surfactant mutant strains. Another reason for the similarity in degradation could be due to the sorption of the surfactants produced by the bacteria to the soil matrix decreasing their effective concentrations. Surfactants produced by bacteria are known to be specific and to emulsify hydrocarbons differently or they may not emulsify hydrocarbons at all. For example, surfactants produced by *Pseudomonas* sp. strain LP1 emulsified diesel, crude oil and engine oil but not pyrene (Obayori et al., 2009). However, in Chapter 3, where the degradation ability of these strains was tested in liquid medium supplemented with diesel as a carbon source, a significant difference in growth was observed between the wild type and mutant strains suggesting that the surfactants were efficient in emulsifying hydrocarbons in a water saturated environment.

From the GC-FID chromatograms, the reduction in peaks and the disappearance of some peaks were the same for all the strains tested indicating that these components are commonly available for utilisation by the tested bacterial strains. All four strains and their surfactant knockout mutants were able to significantly reduce the diesel in soil after 35 days compared to the controls. The presented results are congruent with other reports showing that bacteria are efficient hydrocarbon degraders (Wang et al. 2017). This study, therefore, suggests that these Pseudomonads are suitable for degradation of hydrocarbons.

4.6 Conclusion

In conclusion, epiphytic Pseudomonads and their surfactant mutants were able to degrade different components of diesel but with no significant difference between them. Further research should, therefore, be carried out to understand the interactions between factors

such as the microorganisms, the hydrocarbon to be treated and the surfactants produced by bacteria to achieve effective remediation of hydrocarbons. This will help to elucidate if epiphytic bacteria are good candidates for the remediation of hydrocarbons in terrestrial environments.

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5 Conclusion and future directions

Large amounts of hydrocarbon contaminants are produced during the processing, transportation and utilisation of oil leading to significant hydrocarbon contamination in the soil. Bioremediation uses microorganisms, enzymes and plants to remove these hydrocarbon contaminants from the soil without undesirable side effects. Due to the high hydrophobicity and solid-water distribution ratio, hydrocarbons tend to bind to the non-aqueous phase and organic matter in the soil. Therefore, hydrocarbons become less available for microbial degradation. To increase bioavailability of hydrocarbons, bacteria produce surfactants which enhance the degradation of hydrocarbons. This thesis investigated the roles of surfactants produced by epiphytic bacteria in enhancing the degradation of diesel in soil and how surfactants produced by epiphytic Pseudomonads might support their fitness in the phyllosphere. In this chapter, insights are provided on the findings of this thesis and future directions.

Initially, 21 bacterial strains, selected to represent the diversity of leaf biota, were tested for their ability to utilise diesel and petroleum benzine (Chapter 2). Their ability to produce surfactants was also investigated. Results showed that 40% of the bacterial strains were able to utilise diesel. These bacterial strains, which include Alphaproteobacteria, Gammaproteobacteria and Actinobacteria, were able to grow to high culture density but not to an exponential phase which might be due to the low solubility of diesel in the aqueous medium.

To determine if these epiphytic bacteria can produce surfactants, two screening methods were used i.e., the atomised oil assay and drop collapse assay. The atomised oil assay developed by Burch et al. 2010 is a high-throughput screen used to detect a wide variety of surfactants. It is a semi-quantitative and quicker method of detecting surfactant production in bacteria on agar plates when compared to the drop collapse assay which is labour and time intensive. The drop collapse assay is therefore not suitable for high-throughput screening.

However, in this research the drop collapse assay was able to detect more bacterial strains than the atomised oil assay indicating that the drop collapse assay was more sensitive than the atomised oil assay. Results showed that more than 50% of the epiphytic bacterial strains tested were able to produce surfactants. While nine strains showed surfactant production by producing halos using the atomised oil assay, the drop collapse assay showed that twelve bacterial strains collapsed into oil indicating the presence of surfactants.

While the bacteria that utilised diesel produced surfactants, bacterial strains, such as *Methylobacteruim radiotolerans* 0-.1, *Methylobacterium* sp. Leaf 85 and *Methylobacterium* sp. Leaf 92, which were unable to utilise the diesel also produced surfactants. This is in contrast to other studies that have shown that bacteria from this genus are able to degrade hydrocarbons (Varjani et al. 2018; Efroymson and Alexander 1991; Hasanuzzaman et al. 2007; Salam, Obayori, and Raji 2015; Mishra, Sarma, and Lal 2004). It is unclear why these epiphytic bacterial strains are unable to utilise diesel. As it has shown that, the *Methylobacterium* strains tested all lacked the *AlkB* gene responsible for hydrocarbon degradation. This may explain the reason for the inability to utilise diesel. *AlkB* is an important alkane hydroxylase commonly found in bacteria and is one of the enzymes responsible for aerobic alkane degradation in the bioremediation of oil contaminations (Smits et al. 2002).

Results from Chapter 2 showed an abundance of epiphytic bacteria that produce surfactants in the phyllosphere. Results also support the previous study by Gandolfi et al. 2017 who investigated the commonality of the *AlkB* gene on plant leaves of urban trees and showed that the *AlkB* gene was common among leaf epiphytes. Therefore, these epiphytic bacteria have the potential for use in bioremediation of hydrocarbon contaminants. The presence of alkane degradation genes and surfactant production might be advantageous to their survival on plant leaf surfaces, even though it is unclear if epiphytic bacteria utilise waxes present on the plant leaf surface as a carbon source. Results from this research, therefore, support many studies that have shown that bacteria such as *Acinetobacter*, *Aeromonas*,

Arthrobacter, *Bacillus*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Williamsia* are hydrocarbon utilisers and surfactant producers (Varjani et al. 2017; Efroymson and Alexander 1991; Hasanuzzaman et al. 2007; Salam, Obayori, and Raji 2015; Mishra, Sarma, and Lal 2004; Huang et al. 2008).

The ability of surfactants produced by selected epiphytic Pseudomonads to enhance the colonisation of the plant leaf surface was also investigated. For this study (Chapter 3), the physiological and ecophysiological effects of surfactants in epiphytic Pseudomonads were studied. Firstly, the ability of four epiphytic Pseudomonads to produce surfactants was tested. Results using the two complementary assays (atomised oil assay and drop collapse assay) showed that these Pseudomonads produced surfactants. The surfactants were characterised, and surfactant mutants of these Pseudomonads were obtained. The insertion sites of each mutant were also determined which showed similarities to nonribosomal peptide synthetase (NRPS) in other *Pseudomonas* strains. Effects of these insertions and their loss of ability to produce surfactants were further tested in KB complex medium and M9 minimal medium supplemented with glucose as source of carbon. None of the insertion mutants changed doubling times in the different liquid cultures indicating no impact on fitness effect.

The effect of surfactants produced by epiphytic Pseudomonads on the utilisation of diesel was tested in Bushnell-Haas broth supplemented with diesel as the source of carbon. Results showed that both wild type and mutant strains grew on diesel. In general, the growth rate of the wild type and surfactant mutant strains on diesel was slow when compared to growth in complex or minimal medium supplemented with glucose as sole source of carbon. However, the growth rate of the mutant strains was reduced when compared with the wild type strains. When the mutant strains were supplemented with surfactants extracted from their respective wild type strains or Tween-20 and grown on BHB supplemented with diesel, the surfactants complemented the mutant strain either partially or completely. This shows that the lack of surfactant was responsible for the reduced growth rate.

As stated earlier, surfactant production by bacteria is common in the phyllosphere (Chapter 2). We investigated the ability of surfactant producing-epiphytic Pseudomonads and their non-surfactant producing mutant strains to colonise the phyllosphere (Chapter 3). This was achieved by growing *Arabidopsis thaliana* axenically as described by Miebach et al. 2020. The growth *in planta* of all wild type and surfactant mutant strains were tested individually by inoculating *Arabidopsis thaliana* with respective bacterial strains with an airbrush and sampled for six days. The colony forming unit of each strains and their surfactant mutants determined by growing the strains on LB agar plates (details in chapter 3). From the results obtained, there was no significant difference between the plant colonisation of the wild type and the surfactant mutant strains in their ability to colonise the phyllosphere.

Results obtained from this study did not support the findings of Burch et al. 2014 that showed that surfactants enhance the fitness of bacteria on plant leaf surfaces. This might be due to the fluctuating humidity conditions in which Burch's experiment was carried out (Burch et al. 2014).

The mutants were also co-inoculated with respective wildtype by airbrushing and sampled for six days. The whole above ground plant was measured for six days and colony forming units of the wild type and surfactant mutant strains determined. Results suggested that the wild type and the co-inoculated mutant strains were not statistically significant as the wildtype and surfactant mutants might have benefited from the surfactants produced by the epiphytic Pseudomonads (Burch et al. 2014). This result may also have been influenced by the type of plant used for the study and the condition of growth as previous studies have shown that fluctuating humidity might be a condition to obtain fitness advantage. Inoculating these strains under fluctuating humidity might demonstrate whether surfactant production affects the colonisation of the *Arabidopsis thaliana* plant leaf surface by epiphytic bacteria.

In Chapter 4, the growth potential of surfactant producing epiphytic Pseudomonads on diesel supplemented soil was investigated. Findings from previous chapters in this thesis indicate

that epiphytic bacteria might have potential for oil remediation in the environment. Most past studies have shown that microorganisms utilise hydrocarbons from environments such as soil, water and the atmosphere. For this study, the focus was on bacteria from the phyllosphere. To my knowledge, this is the first research that investigates the effects of surfactants produced by epiphytic bacteria in enhancing the utilisation of hydrocarbons in soil. To achieve this, artificially contaminated soil (soil microcosm supplemented with diesel) was inoculated with bacteria and growth measured over 35 days by determining colony forming units (CFU). Control tubes were also prepared (see Material and Methods in Chapter 4). From the results obtained, the surfactant-producing epiphytic Pseudomonads were able to utilise diesel as the sole source of carbon and energy. There was, however, no significant difference between the wild type and surfactant mutant strains in their utilisation of diesel.

Diesel degradation by epiphytic Pseudomonads in an artificially contaminated soil appeared to occur between days 12-35. The GC-FID analysis of the residual oil content carried out to examine the amounts of diesel removed from the soil, showed that the wild type and mutant strains were effective at removing significant amounts of the components of hydrocarbons. However, the differences between the wild type and surfactant mutant strains in effective hydrocarbon removal were not significant.

In all instances, where there was a comparison of bacterial growth in soil between the wild type and surfactant mutant strains, no significant differences were observed. The wild type strains were expected to utilise hydrocarbons significantly better than the surfactant mutant strains following the results presented in Chapter 3. This deviation may be linked to characteristics of surfactants, the type of hydrocarbon contaminant and the physiological characteristics of the microorganisms involved in the degradation process. (Hua and Wang 2014). Another reason could be the complex interactions that occur between microorganisms, surfactants and hydrocarbons which may result in reduced degradation.

Overall, the results from this thesis showed that there is an abundance of surfactant producers and hydrocarbon degraders in the phyllosphere. The abundance of these epiphytic bacteria make them potential candidates for the bioremediation of hydrocarbon contaminants in the environment. Studies from many researchers have shown that biologically produced surfactants enhance the removal of hydrocarbon contaminants. Investigating the effects of surfactants in the degradation of diesel, knock out strains of surfactant producing epiphytic bacteria were produced. Results obtained showed a significant difference between the wild type and surfactant mutant strains in their ability to degrade diesel in culture medium. In contrast, there was no significant effect in the efficiency of diesel degradation in soil.

5.1 Future directions

Due to increasing concerns regarding oil pollution globally, especially in developing countries, there is a need to find effective methods of remediation. Microorganisms, especially bacteria, have been widely used in this regard and have produced significant results (Ławniczak et al. 2020; Guerra et al. 2018; Tremblay et al. 2017). Many studies have shown that surfactants produced by microorganisms enhanced the emulsification of hydrocarbons (Fenibo et al. 2019; Raaijmakers et al. 2010). Results from this research showed that many epiphytic bacteria are hydrocarbon degraders and surfactant producers. There is a need for future investigations on the characteristics of surfactants that make them important in increasing the bioavailability of hydrocarbons for microorganisms to degrade. There is also a need to study the interactions that occur between microorganisms, surfactants and hydrocarbons in order to limit reduced degradation of hydrocarbons. In future there may be a need to use mixed cultures of bacteria for increased degradation of hydrocarbons.

Future studies should aim at determining if traits of epiphytic bacteria, such as their ability to utilise hydrocarbons and produce surfactants, could enhance their fitness in the phyllosphere. This would involve investigating the ability of these bacteria to utilise the

abundant very long chain aliphates that constitute the cuticular waxes and the cuticle. In addition, investigating the effects of factors such as humidity, leaf type and temperature may enhance our understanding of the role that surfactants produced by epiphytic Pseudomonads play in their survival in the phyllosphere.

This work shows the potential of phyllosphere bacteria to produce surfactant and degrade hydrocarbons. Hence, phyllosphere bacteria should be further studied to explore their potential. In the future, the use of microbial consortia containing different bacterial species i.e. producing different surfactants and catabolic enzymes that enable them to degrade a wide range of hydrocarbons should be considered for bioremediation applications. A hydrocarbon contaminated soil from oil drilling sites could be considered for hydrocarbon degradation experiments. The quantification and identification of hydrocarbons degraded using both GC-FID and GC-MS is another aspect that should be investigated to provide strong analytic platforms capable of generating multiple types of information. These results provide indicators that can be applied for the study of other bacterial species that have biotechnological and bioremediation abilities. It will be useful to screen epiphytic bacteria that can degrade hydrocarbon through high throughput screening method that can detect surfactant producing strains. This could be achieved through sequencing to determine the degradation traits, functional genes or by screening for surfactant production during isolation surveys. Investigating the genetic diversity of these epiphytic bacteria in the phyllosphere will give a broader understanding of the taxonomic and functional variety of these bacteria and access their metabolic potential. Research on genes that control hydrocarbon degradation pathways in bacteria will provide additional insights on the molecular mechanisms and bacterial degradation of hydrocarbons.

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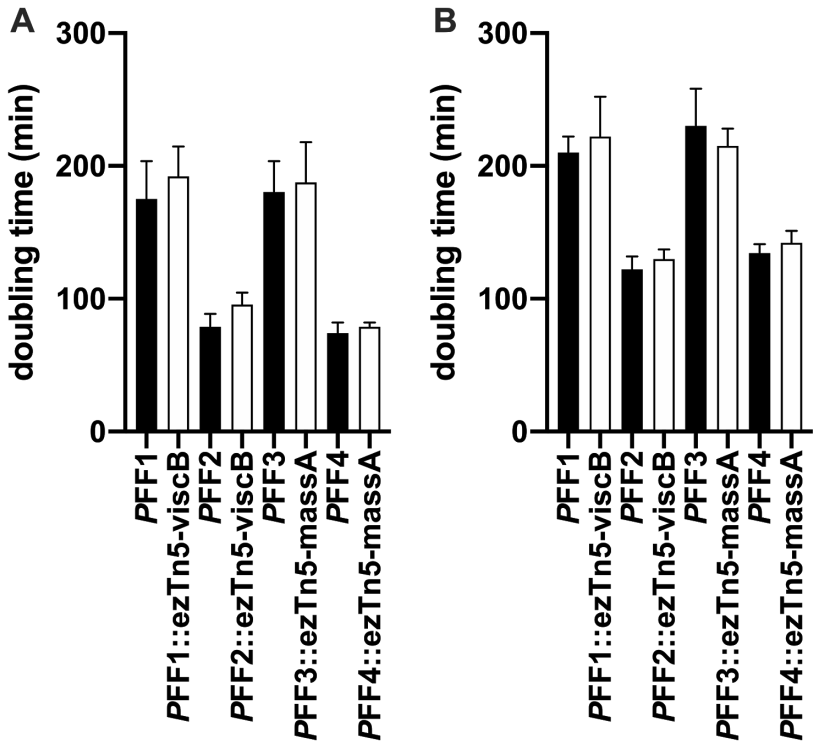
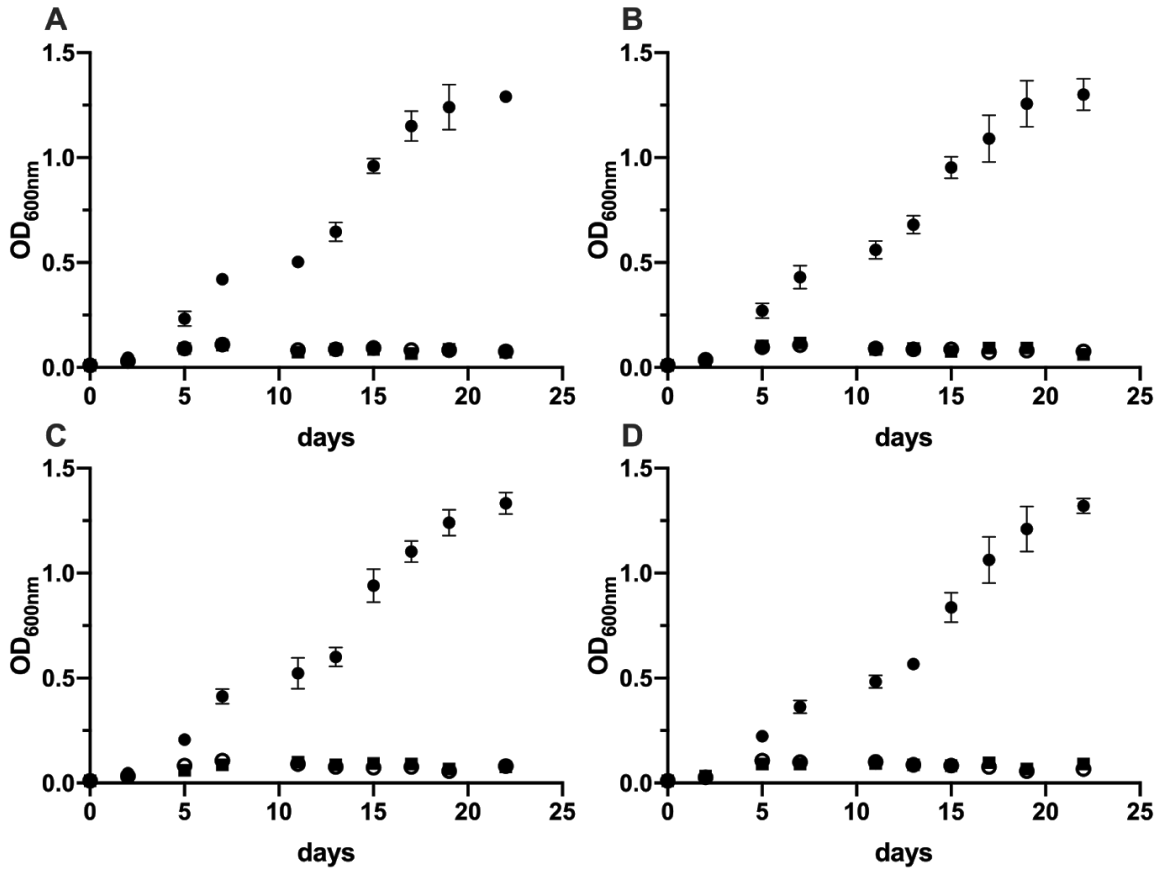
6 Supplemental materials

Supplemental Table 1 Insertion sites of Tn5 transposons.
Tn5 transposon flanking site is underlined.

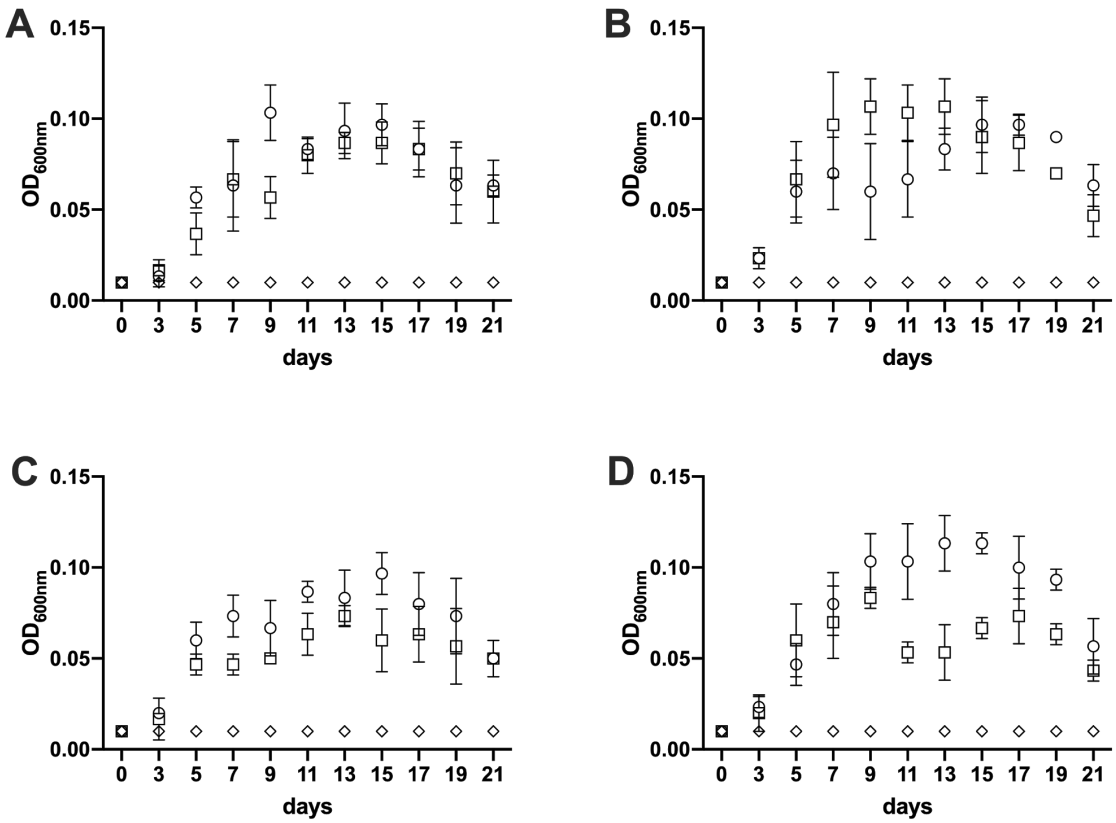
Strain name	Sequence 5' - 3'	Close protein match NCBI accession #
*P*FF1::ezTn5-viscB	AGATGTGTATAAGAGACAGGCGAA CAGGCCCACGAAGCCTTCTTCCGC GACATGCTCGCCGACATCGACGAG CCGACGTTGCCCTTCGGCCTGCAG GACGTGCAGGGCGATGGCCTGGG CATCGACGAAGCGCGCCTGCCGG TTGAGCATGCCCTCAGCCTGCGCT TGCGCAGCCTGGCGCGTCCATTG GGCGTGGGTGTGGCAAGCTTGAT GCACCTGGCGCTGGCCCGGGTGC TGGGGGTGGTGTCGGGCCGGGAG TCGGTGG	AUZ46831.1
*P*FF2::ezTn5-viscB	AGATGTGTATAAGAGACAGATATC ACGGCCGTCGCCCTGCACGTCCT GCACGCCGAAGGGCAAGGTGGGC TCATTGATGTCGGCAAGCATTTCG CGGAAAAACCGCTCATGCTCGTCG ACGCTCACACCCAGGCGCGCCTG GGCCACGTAGTTGCGAAATGGCG CGGCCGCCGGCAAGTCCTGGCCG TGGTTGAACAGGAACGCGCCCATC TCCTGGCCGACCACGTCCATGGC GGTGTGGTCCAGCGCC	QHF44604.1
*P*FF3::ezTn5-massB	AGATGTGTATAAGAGACAGGCTCC ACCTGCAACACCTCGGCCCACAGC CGCGCCAGGGTGGTTTCGACGTC ACCTTGGGGGGCTTGGTATTCACG ACTGAGCAAGGCGCACTGGTCTG GCTGCGGCAGTGCCTTGCGATCCA GTTTTGCC	ABH06368.2
*P*FF4::ezTn5-massB	AGATGTGTATAAGAGACAGGTGAT GCCCTGCCAGGCCGACACCGCCT CGGCATTGGCGGCGGACGCGCTG TGGCGATAGTTGAGCAGTGCGCTG AACAGCGGCGTCGGCGCGAGCAC GCCACTGCAACGCTGGGCCAGGG CCAAGGCCGCGTGTTCATGGCGC AGCAAGGTGGTCAGCCGTGCGTG GGTGGCCTTGACGGCGGTGCGCA CGTCCTGGGCGTCTACGTCCACGC GAAACGGCAAGGTATTG	ABH06368.2



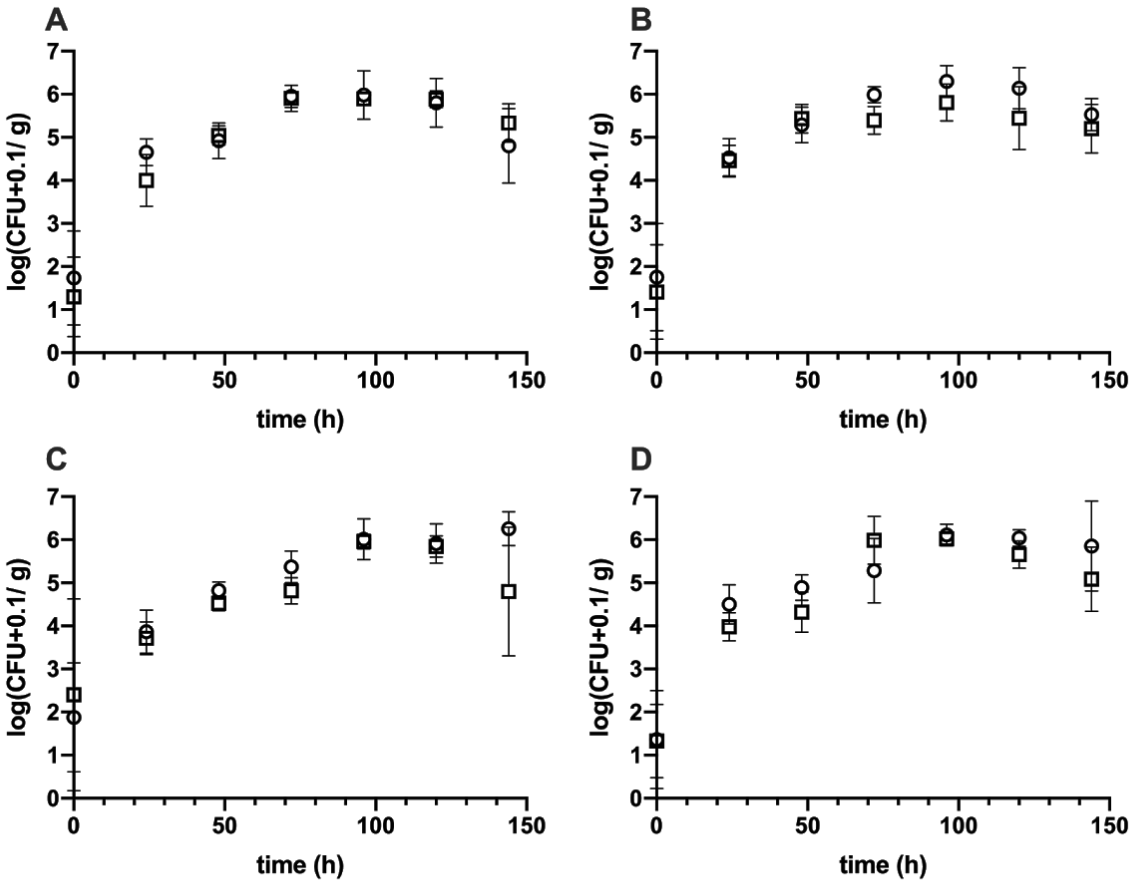
Supplemental Figure 1 Doubling time of wild types and mutants.
On A) KB medium B) M9 supplemented with glucose. No significant difference was found between the doubling time of the wild types and the respective surfactant mutants.



Supplemental Figure 2 Growth of Pseudomonas knockout mutants on surfactants alone. BHB was supplemented with either wild type surfactant (open circles), Tween-20 (filled squares) or diesel as a positive control (filled circles). Growth was only observed on diesel but not on wild type surfactant or Tween-20. A) PFF1; B) PFF2; C) PFF3; D) PFF4. Error bars depict the standard deviation of the mean of three replicates.

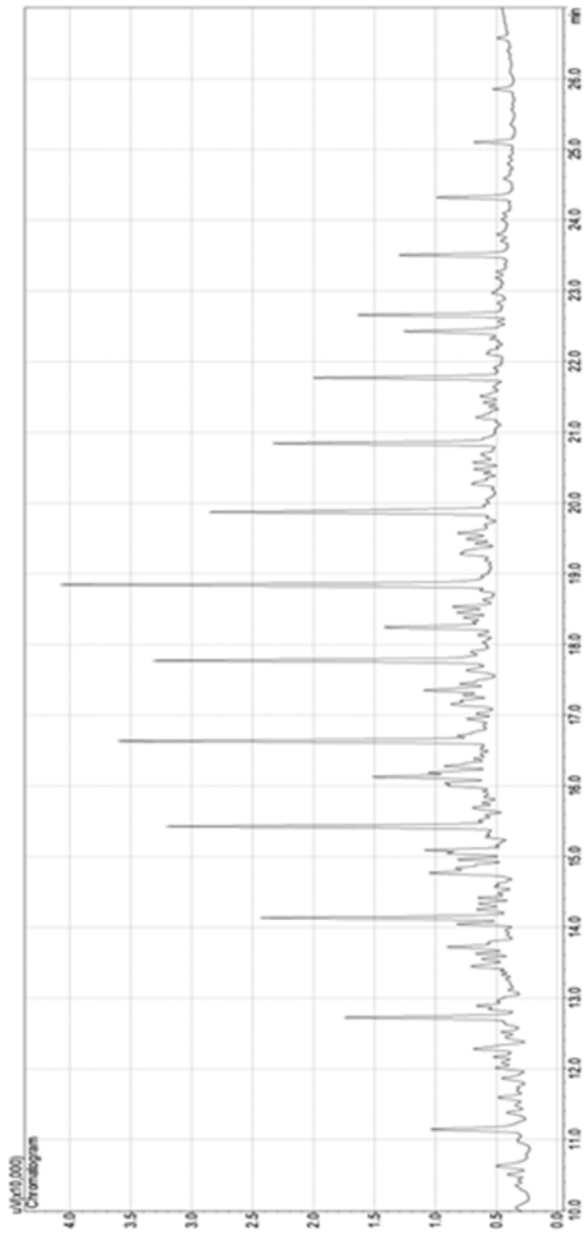


Supplemental Figure 3 Growth of Pseudomonas knockout on dodecane as sole carbon source alone. BHB supplemented with dodecane was inoculated with either wildtype strains (circle), surfactant knockout mutant (square) or was left non-inoculated (diamond). A) growth of PFF1 and respective surfactant mutant. The growth of the surfactant mutant was significantly lower compared to the wildtype on days 5 and 9. B) growth of PFF2 and respective surfactant mutant. From day 7 to day 13, the growth of the surfactant mutant was significantly lower compared to the wildtype. C) growth PFF3 and respective surfactant mutant. The growth of the surfactant mutant was significantly lower compared to the wildtype on days 7, 11 and 15. D) growth of PFF4 and respective surfactant mutant. From day 9 to day 19, the growth of the surfactant mutant was significantly lower compared to the wildtype. The statistical analysis can be found in supplemental table 3. Error bars depict the standard deviation of the mean of three replicates.



Supplemental Figure 4 Growth of wild types and surfactant mutants in planta.
Wild types (open circles) and mutants (open squares). A) PFF1 and PFF1::ezTn5-viscB::, B) PFF2 and PFF2::ezTn5-visB, C) PFF3 and PFF3::ezTn5-massA, D) PFF4 and PFF4::ezTn5-massA. The statistical analysis can be found in supplemental table 4. Error bars depict the standard deviation of the mean of five replicates.

A


uV(x10,000)
Chromatogram
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
min
10.0
11.0
12.0
13.0
14.0
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20.0
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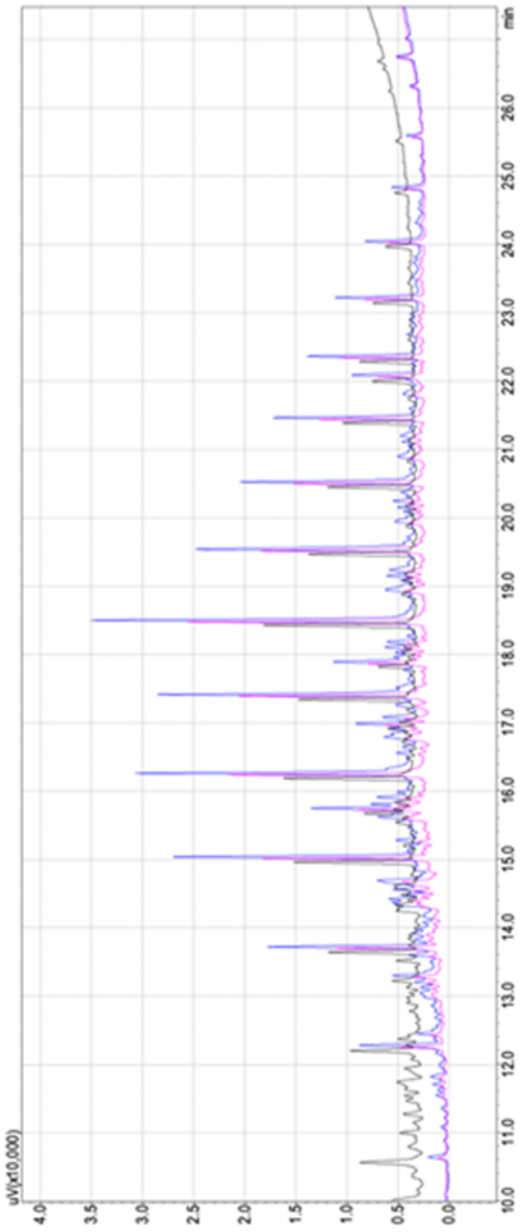
23.0
24.0
25.0
26.0

B


uV(x10,000)
4.0
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2.5
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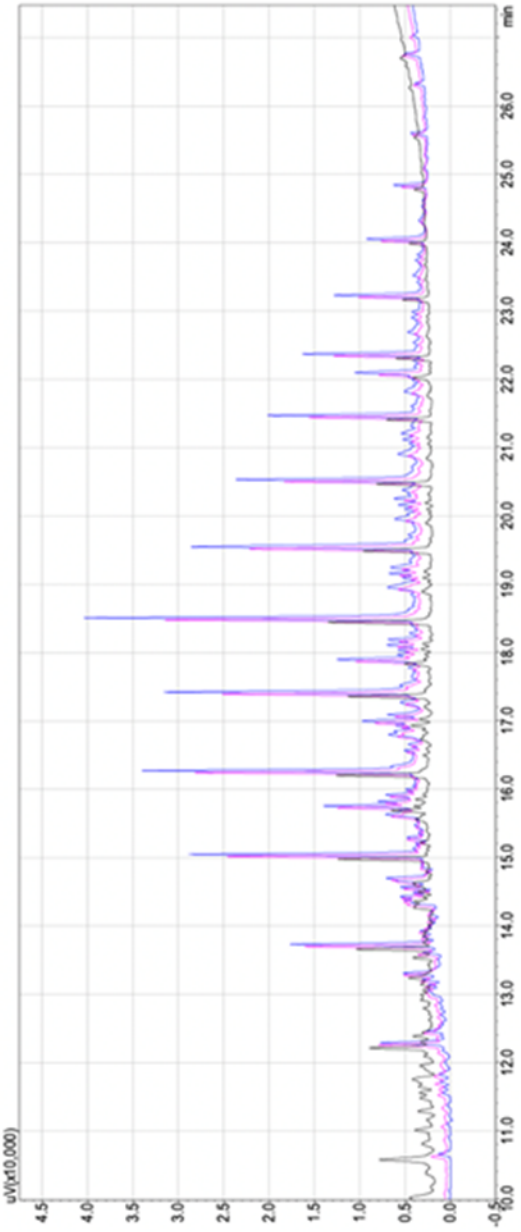
C


uV(x10,000)
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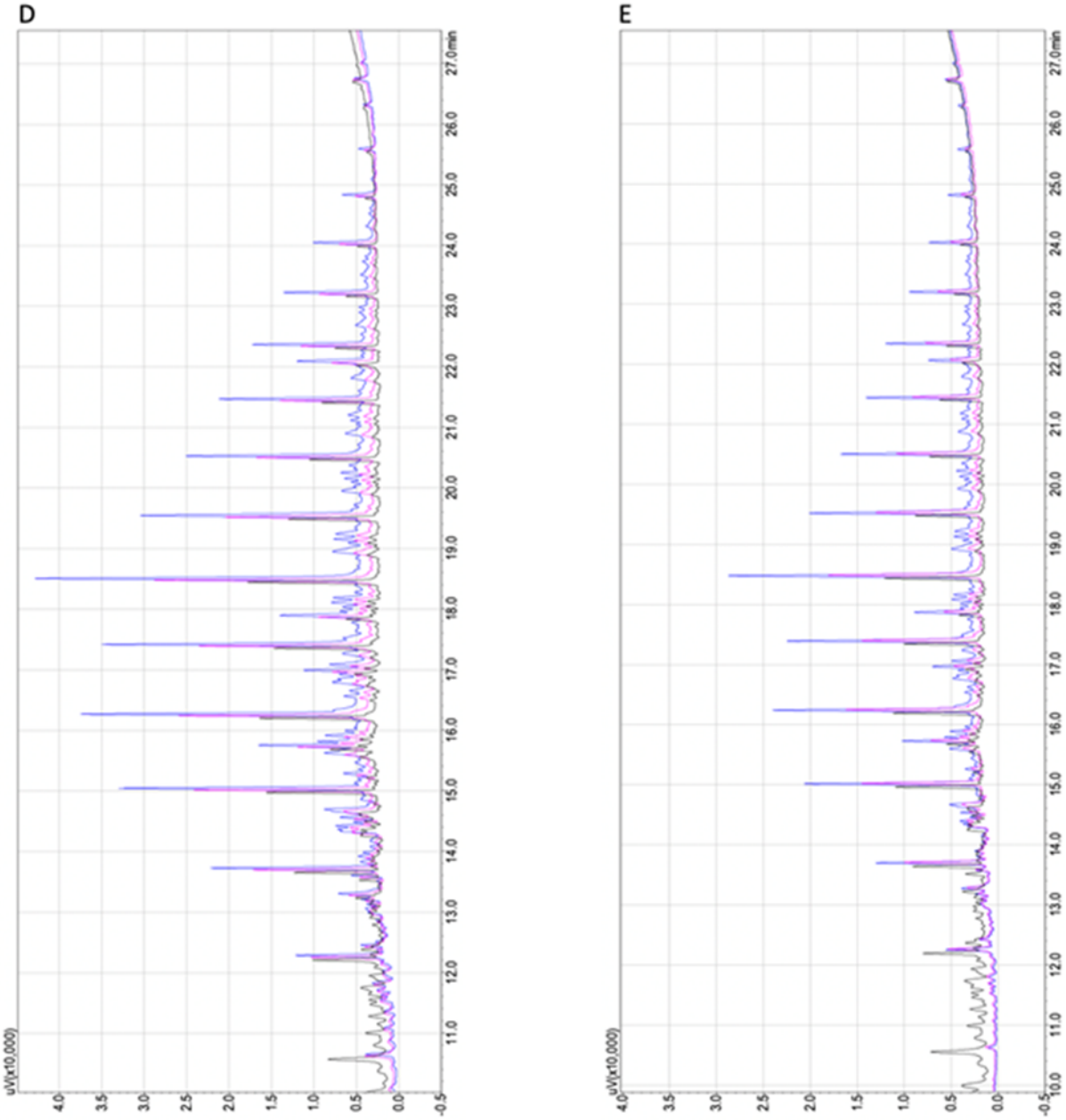

Supplemental figure 5. Gas chromatographic profiles of residual diesel recovered from bacterial degradation in soil microcosms at 0 and 35 days. (A) uninoculated diesel (B) PFF1 (C) PFF2 (D) PFF3 (E) PFF4. Mock treatments are depicted in black, wild type treatments in pink and mutant treatments in blue. Hydrocarbon components of the diesel were degraded by both wild type and surfactant mutant strains at almost the same retention times.

Supplemental Table 2 Result of analysis of nutrients found in soil used in the study of bacterial degradation of diesel.

Properties	Units	Level found
pH	pH Units	5.3
Olsen Phosphorus	mg/L	17
Potassium	mg/kg	129.03
Calcium	mg/kg	3140
Magnesium	mg/kg	102.48
Sodium	mg/kg	41.4
CEC	me/100g	20
Total Base Saturation	%	86
Potentially Available Nitrogen (15 cm depth)	kg/ha	110
Anaerobically Mineralisable	µg/g	623.6
Organic Matter	%	3.6
Total Carbon	%	2.1
Total Nitrogen	%	0.17
C/N Ratio		12.1
Anaerobically Mineralisable N/Total N Ratio	%	3.6

Supplemental Table 3 Statistical analysis of diesel degradation by wild type and the surfactant mutants in soil.
Sidak's multiple comparison test showing the level of significance between the wild type and the surfactant mutants in degrading diesel in soil. No significant difference was found between the strains. There is a significant difference between the individual strains growing on contaminated soil and mock treatment as shown in the table.

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF1 (diesel) - PFF1::ezTn5-nrpsx(diesel)					
Row 1	0.000	-0.5095 to 0.5095	No	ns	>0.9999
Row 2	0.9936	0.4841 to 1.503	Yes	****	<0.0001
Row 3	0.3087	-0.2008 to 0.8182	No	ns	0.5153
Row 4	0.08558	-0.4239 to 0.5951	No	ns	0.9996
Row 5	0.005929	-0.5036 to 0.5154	No	ns	>0.9999
Row 6	0.3385	-0.1710 to 0.8480	No	ns	0.3974
Row 7	0.2625	-0.2470 to 0.7720	No	ns	0.7075
Row 8	0.2862	-0.2233 to 0.7957	No	ns	0.6095

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF1 (diesel) - PFF1					
Row 1	0.000				
Row 2	2.574	0.9845 to 4.163	Yes	*	0.0131
Row 3	2.668	0.7345 to 4.601	Yes	*	0.0263
Row 4	3.014	2.534 to 3.495	Yes	****	<0.0001
Row 5	0.8182	-1.761 to 3.397	No	ns	0.6876
Row 6	1.800	1.171 to 2.429	Yes	**	0.0024
Row 7	2.183	1.783 to 2.583	Yes	***	0.0003
Row 8	2.112	1.733 to 2.491	Yes	***	0.0003

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF2 (diesel) - PFF2::ezTn5-ViscA(diesel)					
Row 1	0.000				
Row 2	0.8657	-1.130 to 2.861	No	ns	0.2491
Row 3	0.4395	-0.09213 to 0.9712	No	ns	0.0812
Row 4	0.07698	-0.7578 to 0.9118	No	ns	0.9976
Row 5	0.3288	0.04097 to 0.6167	Yes	*	0.0312
Row 6	0.09139	-1.634 to 1.817	No	ns	>0.9999
Row 7	0.8981	0.1769 to 1.619	Yes	*	0.0238
Row 8	0.4332	-5.190 to 6.057	No	ns	0.9984

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF2 (diesel) - PFF2					
Row 1	-0.8677	-11.67 to 9.934	No	ns	0.9877
Row 2	1.961	1.133 to 2.789	Yes	**	0.0065
Row 3	1.939	1.281 to 2.597	Yes	**	0.0033
Row 4	2.506	1.386 to 3.626	Yes	**	0.0094
Row 5	2.402	-11.67 to 16.47	No	ns	0.7709
Row 6	1.345	0.5901 to 2.101	Yes	**	0.0065
Row 7	1.633	0.9396 to 2.326	Yes	**	0.0059
Row 8	1.206	-6.274 to 8.685	No	Ns	0.8015

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF3 (diesel) - PFF3 ::ezTn5-lysR (diesel)					
Row 1	0.000				
Row 2	-0.1127	-1.670 to 1.444	No	ns	0.9996
Row 3	1.248	0.9291 to 1.567	Yes	**	0.0018
Row 4	0.8424	-0.4362 to 2.121	No	ns	0.1210
Row 5	0.4572	-2.427 to 3.342	No	ns	0.9894
Row 6	0.3119	-0.5081 to 1.132	No	ns	0.5809
Row 7	-0.05838	-2.163 to 2.046	No	ns	>0.9999
Row 8	0.07035	-0.2484 to 0.3891	No	ns	0.6643

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF3 (diesel) - PFF3					
Row 1	0.000				
Row 2	1.426	0.1138 to 2.739	Yes	*	0.0375
Row 3	2.535	2.173 to 2.896	Yes	***	0.0005
Row 4	2.833	1.102 to 4.564	Yes	*	0.0118
Row 5	1.165	-2.290 to 4.620	No	ns	0.3946
Row 6	1.290	0.5893 to 1.991	Yes	**	0.0056
Row 7	2.008	1.728 to 2.289	Yes	****	<0.0001
Row 8	2.100	1.948 to 2.252	Yes	****	<0.0001

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF4 (diesel) - PFF4 ::ezTn5-massA (diesel)					
Row 1	0.000				
Row 2	0.1441	-0.04251 to 0.3307	No	ns	0.0996
Row 3	-0.1264	-1.418 to 1.166	No	ns	0.9858
Row 4	0.7788	0.3200 to 1.238	Yes	*	0.0132
Row 5	0.8099	-4.342 to 5.962	No	ns	0.8154
Row 6	0.7213	-0.2702 to 1.713	No	ns	0.1101
Row 7	0.1827	-2.175 to 2.540	No	ns	0.9906
Row 8	0.6827	-2.745 to 4.110	No	ns	0.6811

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
PFF4 (diesel) - PFF4					
Row 1	0.000				
Row 2	3.196	2.418 to 3.975	Yes	**	0.0019
Row 3	2.669	1.268 to 4.069	Yes	*	0.0131
Row 4	1.615	1.451 to 1.780	Yes	****	<0.0001
Row 5	3.459	3.342 to 3.576	Yes	****	<0.0001
Row 6	3.894	3.156 to 4.632	Yes	***	0.0004
Row 7	2.078	1.476 to 2.681	Yes	**	0.0034
Row 8	2.032	1.835 to 2.229	Yes	****	<0.0001

Appendix 1. Bacteria used in study and their genome accession numbers

The table below shows the bacteria used in the study and their genome accession numbers. The EPA import permit for the epiphytic bacteria was NOC 100168.

Bacteria used in study (chapter 2) and their accession numbers

Strain	Genomes accession number
***Acidovorax* sp. Leaf 84**	LMMC00000000
***Aeromicrobium* sp. Leaf 245**	LMMD00000000
***Agreia* sp. Leaf 335**	NZ_LMOQ00000000
***Arthrobacter* sp. Leaf 145**	LMON00000000
***E. coli* DH5α**	NZ_JABFON010000001
***M. radiotolerans* 0–1**	NR_036824
***Methylobacterium* sp. Leaf 85**	LMME00000000
***Methylobacterium* sp. Leaf 92**	LMMQ00000000
***Methylophilus* sp. Leaf 414**	NZ_LMQQ00000000
***Microbacterium* sp. Leaf 320**	LMOD00000000
***Pantoea agglomerans* 299R**	ANKX00000000
***Plantibacter* sp. Leaf 1**	LMJX00000000
***P. citronellolis* P3B5**	NZ_CP014158
***P. syringae* B728A**	NZ_QJTV00000000
***Rathayibacter* sp. Leaf 296**	LMNR01000000
***Rhodococcus* sp. Leaf 225**	LMRN00000000
***Sphingomonas melonis* Fr1**	NZ_ATTG00000000
***Sphingomonas phyllosphaerae* FA2**	NZ_ATYK00000000
***Sphingomonas* sp. Leaf 17**	LMKL01000000
***Sphingomonas* sp. Leaf 34**	LMLC01000000
***Sphingomonas* sp. Leaf 357**	LMPM01000000
***Williamsia* sp. Leaf 354**	LMPL00000000

Various mineral salt medium used in testing the ability of epiphytic bacteria to utilise diesel.

In chapter 2, various minimal media were tested to determine the utilisation of diesel by epiphytic bacteria. These various media supplemented with diesel did not support the growth of the epiphytic bacteria. The bacteria however grew in media supplemented with different sugars according to their nutritional demands (see table 2-1). Details of the hydrocarbon utilisation assay is found in chapter 2.

Mineral salt medium (Kastner Breuer-Jammali and Mahro, 1994)

Mineral salt medium	g/L
Na_2HPO_4	2.13
KH_2PO_4	1.30
NH_4Cl	0.50
$MgSO_4.7H_2O$	0.20
pH	7.0

Sterilize by autoclaving for 15 minutes at 121º C

Carbon free mineral salt medium (Habe et al., 2002)

Carbon free mineral salt medium	g/L
NH_4NO_3	3.0
Na_2HPO_4	2.2
KH_2PO_4	0.8
$MgSO_4.7H_2O$	0.1
$FeCl_2.6H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Yeast extract	0.005

Sterilize by autoclaving for 15 minutes at 121°C

Trace-element solution (Widdel and Pfennig. 1981)

Trace element solution	g/l
$NaNO_3$	4
KH_2PO_4	1.5
Na_2HPO_4	0.5
$FeSO_4 \cdot 7H_2O$	0.0011
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.01

Sterilize by autoclaving for 15 minutes at 121^{o}C

References

Käestner M., M. Breuer-Jammali and B. Mahro, 1994. Enumeration and Characterization of the Soil Microflora from Hydrocarbon-Contaminated Soil Sites Able to Mineralize Polycyclic Aromatic Hydrocarbons. *Applied. Microbiology and Biotechnology*. 41: 267–273.

Widdel F., N. Pfennig. 1981. Studies on Dissimilatory Sulfate-Reducing Bacteria that Decompose Fatty Acids I. Isolation of New Sulfate-Reducing Bacteria with Acetate from Saline Environments. Description of *Desulfobacter postgateigen*. nov., sp. nov. *Archaea Microbiology*. 129: 395

Habe H., Ashikawa, Y., Saiki, Y., Yoshida, T., Nojiri, H., and Omori, T. 2002. *Sphingomonas* sp. strain KA1, Carrying Acarbazole Dioxygenase Gene Homologue, Degrades Chlorinated Dibenzo-p-dioxins in soil. *FEMS Microbiology Letters*. 211:43–49.

Table showing diesel utilisation in various minimal medium by epiphytic bacteria.

Strain	Mineral salt medium	Carbon free mineral salt medium	Basal salt medium	Mineral salt medium
***Acidovorax* sp. Leaf 84**	-	-	-	-
***Aeromicrobium* sp. Leaf 245**	-	-	-	-
***Agreia* sp. Leaf 335**	-	-	-	-
***Arthrobacter* sp. Leaf 145**	-	-	-	-
E. coli DH5α	-	-	-	-
***M. radiotolerans* 0–1**	-	-	-	-
***Methylobacterium* sp. Leaf 85**	-	-	-	-
***Methylobacterium* sp. Leaf 92**	-	-	-	-
***Methylophilus* sp. Leaf 414**	-	-	-	-
***Microbacterium* sp. Leaf 320**	-	-	-	-
***Pantoea agglomerans* 299R**	-	-	-	-
***Plantibacter* sp. Leaf 1**	-	-	-	-
***P. citronellolis* P3B5**	-	-	-	-
***P. syringae* B728A**	-	-	-	-
***Rathayibacter* sp. Leaf 296**	-	-	-	-
***Rhodococcus* sp. Leaf 225**	-	-	-	-
***Sphingomonas melonis* Fr1**	-	-	-	-
***Sphingomonas phyllosphaerae* FA2**	-	-	-	-
***Sphingomonas* sp. Leaf 17**	-	-	-	-
***Sphingomonas* sp. Leaf 34**	-	-	-	-
***Sphingomonas* sp. Leaf 357**	-	-	-	-
***Williamsia* sp. Leaf 354**	-	-	-	-

All media listed in table did not support the growth of bacteria (-).

Appendix 2. Effects of the biosurfactants from phyllosphere colonising Pseudomonad and its effect on plant colonisation and diesel degradation

In chapter 3, the effects of surfactants produced by epiphytic bacteria in enhancing the degradation of hydrocarbons and determining the fitness of bacteria in the phyllosphere were tested. To this end a Tn5 random transposon mutagenesis using conjugation was carried out to generate surfactant knockout mutants for *Pseudomonas citronellolis* P3B5 which has been shown in chapter 2 to produce surfactants and utilise diesel. Mutants with phenotypes different from the wild type strains were tested using the atomised oil assay and drop collapse assay as described in chapter 2. To locate the transposon insertions in the mutants, an arbitrary PCR as described by Das et al., 2005 and Schlechter and Remus-Emsermann, 2019 was carried out.

Random transposon mutagenesis

Materials and method

The donor strain *E. coli* S17-1 (pMRE-Tn5-145) and recipient strain (*Pseudomonas citronellolis* P3B5) were grown on Lysogeny broth agar (LBA, Oxoid) and Lysogeny broth (LB, Oxoid) at 37º C or 30° C, respectively, S-Pak membrane filter paper was used to grow donor and recipient strains on LB agar. Gentamicin was used at working concentration of 15 µg/ml. Conjugants were grown on M9 agar containing gentamicin to counter select the donor strain (M9 agar supplemented with 20 % glucose and containing gentamicin will select for successful transposon integration events).

Method

Conjugation

Overnight cultures of donor and recipient strains were produced by growing them individually in a flask containing 50 ml of LB broth containing gentamicin or LB broth, respectively.

Flasks were incubated at 30º C and 37º C. On the second day, fresh liquid cultures were prepared from the overnight liquid cultures i.e. 50 µl of overnight liquid culture was added to 5 ml of LB broth containing 5 µl of gentamicin and 50 µl of overnight culture of *Pseudomonas citronellolis* P3B5 was added to 5 ml of LB broth. Both tubes were incubated in a shaking incubator at 37º C and 30º C respectively and allowed to reach an OD600 of 0.6 before harvesting. To harvest the strains, the cultures were centrifuged at 6000 × *g* for 10 mins and washed twice in 5 ml of 1 × PBS before resuspension in 300 µl of 1 × PBS. The donor and recipient were diluted to similar amounts and optical density and mixed at ratio 1:1. The mixture of donor and recipient cells was grown on a S-Pak membrane filter paper, 47 mm diameter, with a pore size of 0.45 µm filter paper placed on a LB agar plate and incubated overnight at 37º C. On the third day, the filter containing the conjugation mixture was placed in a 15 ml falcon tube containing 500 µl of 1 × phosphate buffer saline (PBS, 0.2 g L^{-1} NaCl, 1.44 g L^{-1} Na_2HPO_4 and 0.24 g L^{-1} KH_2PO_4) and vortexed until the biomass is suspended. 100 µl of the mixture was grown on M9 agar containing gentamicin and glucose as source of carbon. 100 µl of a ten-fold dilution series up to 10^{-2} were also grown on M9 agar containing gentamicin and glucose as source of carbon to select the donor strain (85.1 g L^{-1} $Na_2HPO_4.2H_2O$, 30 g L^{-1}, 5 g L^{-1} NaCl, 10 g L^{-1} NH_4Cl, 1 M $MgSO_4$, 0.1 M $CaCl_2$, 3 % agar, 20 ml of 20% glucose was added as carbon source). Plates were incubated at 30º C. Plates were checked for colonies after 48 hours.

Arbitrary PCR

A small loop of bacteria was resuspended in 100m µl of 50 mM NaOH and incubated at 95 ºC for 15 minutes. Debris was removed by a brief centrifugation and 1 µl of the supernatant was used as a template for the subsequent PCR.

The first round of PCR was carried out in 20 µl total volume using KAPA2G PCR mix (taq polymerase) and three arbitrary primers Arb1, Arb2, and Arb3 and the transposon specific primer ARB-RB-PCR1.

Primers used in study

Name	Sequence 5’ to 3’
Arb1	GGCCACGCGTCGACTAGTCANNNNNNNNNNGCTCG
Arb2	GGCCACGCGTCGACTAGTCANNNNNNNNNNGACTC
Arb3	GGCCACGCGTCGACTAGTCANNNNNNNNNNGATAC
ARB-RB-PCR1	ctggggtaatgactctctagc
ARB-RB-PCR2	ctgagtaggacaaatccgccg
PCR2 AP-PCR	GGCCACGCGTCGACTAGTCA

Mixed arbitrary primers - (ARB 1+2+3), ARB 4

Specific primer - ARB-RB-PCR1

Reaction mix (20 µl total reaction volume)

KAPA2G	10 µl
Specific primer	1 µl
Mixed arbitrary primers (10mM)	1µl
DNA template	1 µl
Water	7 µl

PCR was performed with the following settings:

98° C for 3 min

95° C for 15 sec

30° C for 20 sec

72° C for 30 sec -> go back to step 2 six times

95° C for 15 sec

45° C for 20 sec

72° C for 30 sec -> go back to step 5 30 times

72° C for 1 min

10° C for ∞

Run 5 µl on 1 % agarose gel

Clean up using INtRON biotechnology Megaquick-spin total fragment DNA purification kit

100 µl of BNL buffer was added to 20 µL PCR reaction tube directly and mixed. A mega spin column was placed in a collection tube for each reaction. The DNA mixture was dispensed into the column and centrifuged at 12000 × *g*. 1 minute. The residue was decanted and 700 µl of washing buffer was dispensed into the column and centrifuge at 13000 × *g* for 1 minute. The residue was decanted, and the column was dried by centrifugation at 18928 × *g* for 1 minute. The residue that was recovered was decanted. The column was placed in a 1.5 ml microcentrifuge tube and 50 µl of sterile distilled water was added into the column and centrifuged at 13000 rpm for 1 minute to elute the DNA.

ARB PCR reaction 2

A Master mix was prepared using KAPA2G PCR mix, DNA template (DNA fragment recovered after clean-up) and suitable primers combination was prepared for 2nd PCR

Primer set used - ARB-RB-PCR 2

PCR2-AP-PCR

Reaction mix (20 µl total reaction volume) was prepared.

KAPA2G 2X master mix	10 µl
Specific primer	1 µl
Arbitrary primer	1 µl
DNA template	1 µl
Water	7 µl

PCR reaction was used to perform a PCR with the following settings.

95º C for 3 minutes

95º C for 15 secs

58º C for 10 sec

72º C for 30 secs – go back to step 2 31 times

10º C for∞

5 µL of the PCR reaction were loaded and analysed ran on 5 ul on 1% agarose gel.

Clean up using Zymo research DNA clean up and concentrator

100 ul of binding buffer was added to 20 ul fragment DNA and mixed. The mixture was transferred into a column placed in a collection tube and centrifuge at 10000 × *g* for 30 seconds. The flow through was discarded. 200 ul of DNA wash buffer was added to the column and centrifuged at 10000 × *g* for 30 seconds. The wash step was repeated. 10 ul of water was added to the column and incubated at room temperature for 1 minute. The column was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 30 seconds to elute the DNA. Concentration was determined using nanodrop. To send for sequencing, 5 µl of primer (ARB-RB-PCR2) was added to 5 µl of DNA.

Results showed that mutants were successfully generated however, no surfactant mutant were produced.

Reference

Das S., J. C. Noe, S. Paik and T. Kitten. 2005. An Improved Arbitrary Primed PCR Method for Rapid Characterization of Transposon Insertion Sites. *Journal of Microbiological Methods*. 63: 89-94.

Schlechter R. O., and Remus-Emsermann M. N. P. 2019. Delivering "Chromatic Bacteria" Fluorescent Protein Tags to Proteobacteria Using Conjugation. *Bio-protocol*. 9(7):e3199.

Appendix 3. Determining the utilisation of waxes present in the cuticle by epiphytic bacteria.

Experiment to determine the utilisation of waxes present in the cuticle by epiphytic bacteria.

This experiment was carried out to determine the ability of epiphytic bacteria to utilise waxes on the plant leaf surface. The epiphytic bacteria used in this study are hydrocarbon degraders and therefore might utilise the components of waxes present on the plant leaf surface as sources of carbon. This experiment was stopped due to the COVID-19 pandemic.

Methods and Materials

Choice of plant

Waxes was extracted from the leaves of *Griselinia littoralis*, a native plant species commonly known as New Zealand broadleaf. This species was chosen as it was readily available on the University of Canterbury campus, and because upon visual observation, the leaves seem to have a relatively thick waxy cuticle.

Choice of bacteria

The bacteria used in this study are members of the genus *Pseudomonas* i.e. *Pseudomonas* sp. FF1 and *Pseudomonas citronellolis* P3B5. *Pseudomonas* spp. were chosen as they are common colonisers of leaf surfaces and their ability to utilise hydrocarbons (see chapters 2, 3 and 4). Also they possess a diverse metabolic capability and can adapt to the fluctuating environmental conditions in the phyllosphere.

Solvent and wax extraction

The solvent of choice for leaf wax extraction was chloroform. Leaves were placed into beaker containing 50 ml chloroform and left for 30 seconds under gentle agitation. This procedure allowed for chloroform-soluble compounds to be extracted without disruption of

the leaf interior. The chloroform extracts were evaporated at room temperature (approx. 25 °C) under a fume hood until the solvent has fully evaporated, leaving the solid wax residue in the beaker. The waxes were then cleaned by dissolving the extracted wax in a known volume of chloroform and filtered using No. 1 Whatman filter paper, to remove dirt and other solid contaminants. The solvent was evaporated off as above and the wax was dissolved in chloroform in a 50 ml falcon tube and the same volume of milli-Q water was added (1:1 ratio). The falcon tube was placed on a vortex for approximately 10 minutes, then removed and left for the liquid layers to separate. The water layer was removed, and the remaining layer was left at room temperature for the chloroform to evaporate, leaving purified epicuticular wax with contaminants such as dirt and water soluble sugars removed. This step of using water to remove sugar is important, as the bacteria will utilise sugar before long hydrocarbons compounds in wax, so it is crucial to remove as much sugar as possible.

Preparation of bacteria and media

P. sp. FF1 and *P. citronellolis* P3B5 were streaked onto separate Lysogeny broth agar plates. A colony of bacterium was used to inoculate a 5 ml Lysogeny broth, which was grown to saturation in a 30°C shaking incubator.

Experimental set-up to determine the utilisation of waxes by Pseudomonads

The experiment comprised five different treatments for both *P.* sp. FF1 and *P. citronellolis*. Two of the five treatments had three replicates. For each species there were nine test tubes (size of tubes were suitable for measuring with a spectrophotometer).

The treatments were: 1) glucose only, 2) no carbon source, 3) wax and surfactant (Tween-20) (three replicates), 4) only surfactant, and 5) only wax (three replicates). Also, a negative control tube containing wax and not supplemented with bacteria was used to measure the time required to wait after shaking tubes, prior to taking OD readings (wax will likely be solidified flakes and contribute to a higher OD). A dilution series in 1 × PBS was used to dilute the saturated liquid cultures of bacteria 100-fold.

A 0.1% (w/v) concentration was used for the wax, glucose, and surfactant; for example, in 5 ml of liquid medium, 5 µl of surfactant, glucose, or wax (5 mg) was added. Cultures were covered tightly with tinfoil and kept in a shaking incubator at 30 °C. To measure the growth of bacterium in the liquid medium, (OD600) of the cell densities was measured every two days.

Gas chromatography-flame ionisation detection (GC-FID) & gas chromatography-mass spectrometry (GC-MS)

In the future, GC-FID will be used to observe any differences in the composition of the wax prior to and after exposure to *P.* sp. FF1 and *P. citronellolis* P3B5. If there is a difference in composition, the results should indicate how significant the difference is.

GC-MS will be used to obtain a more detailed chemical analysis and will provide information about which specific compounds are being degraded (and possibly utilised) by the Pseudomonads when comparing samples from before and after addition of bacteria.